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# Development of a PCR assay to quantify take-all pathogens of wheat

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A thesis submitted in partial fulfilment  
of the requirements of the Open  
University for the degree of Doctor of  
Philosophy

2005

Harper Adams University College

DATE OF SUBMISSION 14 SEPTEMBER 2005  
DATE OF AWARD 28 NOVEMBER 2005

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This PhD is dedicated to my Grandparents who could not see me get this far, I am grateful for all their love and support.

Vyvyan and Gillian Watts-Jones

Richard and Betty Wilson

## Acknowledgements

There are so many people who helped me to get this position that I am sure that I will forget someone but here goes;

The work was sponsored by Agrovista Ltd, NRM Ltd, and Harper Adams University College.

Thank you to Geoff Bateman of Rothamsted Research, UK for providing the take-all isolates.

Thanks to the agronomists who helped me find sites and, farmers who let me use their fields.

My supervisors have guided and supported me with amazing patience and understanding.

Thank you Simon for enlightening me on the topic molecular biology and many others!

Pete thanks for encouraging me to keep going!

Thanks to the CERC team who were always happy to answer my questions!

The PhDs at Harper have always been there to listen to my problems.

In particular, I would like to thank Al, Ian, Rachel and Alison for helping me in the field – great deal of patience is needed for that. Rumi for watering my plants and providing the eyespot isolates. Matt, Jackie and Heidi for helping me find my way through the maze that is doing a PhD. And everyone else for the drinks, chats and etc!

Thanks to my friends; Em who thinks I am crazy for doing a PhD but listens anyway;

Nicki and Peter for being so much more than a landlord and landlady.

My family have always been there for me in the good and the bad times. Sometimes it hasn't been easy particularly when rain is soaking through your coat and shoes. Dad and Jo, thanks for the digging of holes and pulling up of plants.

It hasn't been easy to stay sane and well while doing this and couldn't have done it without any of you.

THANK YOU!

## Abstract

Take-all is a disease in wheat which can cause significant yield losses. If it was possible to predict these losses, by competitive PCR, agronomic decisions could be made to reduce them. As the causal agent, *Gaeumannomyces graminis*, is found in the soil organic matter, soil DNA extraction methods were tested for their efficacy. An inhibition assay was designed to test the level of inhibition present in DNA extracted from soil. Of the extractions tested the CTAB extraction was the most successful at removing inhibitors and the commercially available Soil DNA Isolation Kit <sup>TM</sup> was the least successful. All extraction methods tested required some dilution to enable the amplification of the inhibition standard. Soil DNA extraction was found to be time consuming so stem bases and roots were used with the competitive PCR assay.

To create a quantitative competitive PCR assay published primers were amplified with known *G. graminis* isolates. They produced unexpected results which meant that they were unsuitable for use in a competitive PCR assay. Consequently HAGG primers were designed and successfully amplified known *G. graminis* var. *tritici* and var. *avenae* isolates, and isolated *G. graminis* from take-all infected wheat plants. The HAGG competitive PCR assay was used to quantify the presence of the take-all pathogen in a large number of plant samples ranging in age. Regressions of DNA concentration and visual symptoms or yield did not result in strong correlations. Analysis suggested the variability between the fields explained a large proportion of variability.

A large scale experiment was completed to compare the impact of various seed treatments on pathogen DNA concentration, visual disease assessment, grain quality and yield. Early assessment of the plant material demonstrated the heterogeneous nature of this disease. When taking the pathogen DNA concentration at stubble into account a Latitude® (silthiofam) seed treatment achieved the greatest yield; Baytan® (triadimenol and fuberidazole) resulted in the lowest yield. The experiment demonstrated that this competitive PCR assay has the potential in field experiments to take account of the initial inoculum load.

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# **Chapter One**

## **Literature Review**

## 1.1 Take-all

Take-all is the name given to a disease observed in cereals which causes premature bleaching of the ears known as whiteheads, and stunting and blackening of stems and roots. Four varieties of the causal pathogen *Gaeumannomyces graminis* (*tritici*, *avenae*, *graminis* and *maydis*) have been observed to cause this disease to varying degrees on various plant species. *Tritici* (*Ggt*) is mainly associated with wheat (*Triticum aestivum* L.) where it can cause significant damage, but it also infects barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) (Shou, 1981). *Tritici* is considered to cause the most severe disease on wheat and the least on rye, with intermediate affects on barley and triticale (a hybrid of wheat and rye) (Gutteridge *et al.*, 1993). The variety *avenae* (*Gga*) causes disease in oats (*Avena sativa* L.), although it can also be found on other cereals whilst *graminis* (*Ggg*) is a pathogen of rice (*Oryza sativa* L.) and grasses (Scott, 1981). The recently identified variety *maydis* (*Ggm*) has been shown to cause the disease in maize (Yao, 1992).

It appears that take-all is present worldwide, in all regions where cereals or grasses are grown (Hornby, 1998a). From the first recorded observations of take-all on wheat in the 19<sup>th</sup> Century, the severity of the disease on wheat and yield losses have varied from year to year. With the increased intensification of farming, the occurrence of the disease appears to have risen to a point in the 1980s where it caused more financial loss to UK agriculture than any other disease, suggested to range from £16 to £55 million from year to year (Hornby, 1998a). Studies have shown take-all symptoms in 44% of fields, indicating the high prevalence of take-all in Germany, France and the UK in recent years (Becker *et al.*, 1998). Hardwick *et al.* (2001) showed that between 1989 and 1998 take-all was seen to be widespread throughout the UK.

Symptoms of take-all can appear as early as the seedling stage where the characteristic black roots are observed. A reduction of the root systems often occurs. The effects of take-all can be seen in the field later in the growing season by premature ripening



of the ears, described as whiteheads (Figure 1.1). Grain quality is reduced because they can be shrivelled and smaller than their healthy counterparts and stunting of plants is also indicated as a symptom (Clarkson and Polley, 1981). These symptoms are probably due to the invasion of the stele of the roots of the plant (Liu *et al.*, 2000) which inhibits the flow of water and nutrients to the rest of the plant reducing its ability to grow. Yield reduction tends to be greatest after two or more successive years of the same crop (Rosser and Chambers, 1968). Yield loss has been linked to take-all symptoms. Severe take-all, where 75% of the roots of a plant were blackened, were associated with grain yield losses of between 36 to 66% (Polley and Clarkson, 1980). Yield losses may not be uniform across a field, as a characteristic of take-all is its patchy distribution (Gams and Domsch, 1969). This patchy distribution is due to the fungus spreading from plant to plant by the growth of mycelium along roots (Hornby, 1998a).

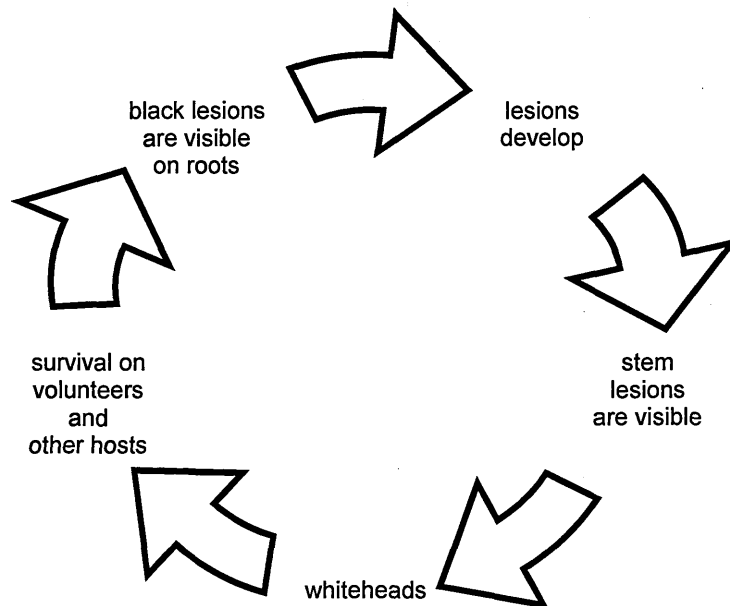


Figure 1.1: Cycle of the symptoms of take-all (adapted from Hornby, 1998a)

### 1.1.1 Taxonomy

The current thinking is that the fungi from genus *Gaeumannomyces* belong to the Family Diaporthaceae in the Order of Diaporthales (Hornby, 1998a). In the past the genus was known as *Ophiobolus* until it was reclassified in 1952 (von Arx and Olivier), however for a number of years some authors still referred to the take-all fungus as *O. tritici*. Recently, DNA sequencing has shown that *G. graminis* appears to be closely related to *Magnaporthe* sp. (Bryan *et al.*, 1995). Consequently, it has been suggested that *Gaeumannomyces* spp. should be reclassified, again, as members of the Magnaporthaceae family (Hornby, 1998a).

Von Arx and Olivier (1952) described isolates of *Ophiobolus graminis* as producing spherical perithecia of between 300 and 500 µm in diameter. The necks were seen to be approximately 160 to 250 µm long with a diameter of 180 µm. They narrowed towards the end protruding from the media. The angle of the neck is almost vertical from the main part of the perithecia. The cylindrical or club-shaped asci form on stalks of various lengths, and contain eight spores. The spores are blunt ended and hyaline, containing oil droplets in early development. At maturity they usually have five septa and are between 65 and 95 µm in length by 3 to 4 µm wide. Therefore, the authors believed it to be a scolecosporus Diaporthales rather than Pseudosphaeriaceae. Other Diaporthales species produce perithecia which have necks which protrude out of the medium at right angles to the main body, or they produce spores which are shorter and have less septa. As a result a new genus within Diaporthales was proposed with the name *Gaeumannomyces*. They suggested that *Ophiobolus graminis* Sacc. and *Rhaphidophora graminis* Sacc. were in fact *Gaeumannomyces graminis*, which caused the disease known as take-all in a number of hosts.

Weste and Thrower (1963) determined that *Ophiobolus graminis* produced ascospores on a media with a specific ration of carbohydrates and nitrogen. The maximum number of perithecia were produced when the media contained 0.2% asparagine and 1 - 1.5% glucose, or 0.4% asparagine and 2% glucose. In addition to the ascospores, falcate microconidia were produced on this media. These spores were about 5 by 2  $\mu\text{m}$  in size, although the authors were unable to germinate these spores.

Fungi isolated from wheat appeared to produce spores that were 101 to 117  $\mu\text{m}$  long whereas those isolated from oats produced ascospores of 79 to 86  $\mu\text{m}$  long. It was suggested that those isolates that were found to be infecting oats should be classed as a different variety, *Ophiobolus graminis* Sacc. var. *avenae* E. M. Turner (Turner, 1940).

Work completed by Walker in 1972 attempted to address issues regarding the naming of the fungi that infect plants causing take-all. The work supported that carried out by von Arx and Olivier in 1952. It was determined that *O. oryzinus* Sacc. and *G. graminis* (Sacc.) Arx and Olivier were the same and, suggested that they be known as *G. graminis* var. *graminis* (Ggg). This variety produced mycelium with lobed brown hyphopodia on its host. Later this variety was seen to produce melanized hyphopodia or appressorium on aerial parts of plants (Epstein *et al.*, 1994). It is known to produce 1, 8-dihydroxynaphthalene (DHN) melanin in the cell walls of its mycelium and hyphopodia. Mutants that did not produce melanin appeared to show less virulence (Frederick *et al.*, 1999). In contrast those isolates associated with wheat appeared to have simple unlobed hyphopodia and consequently were given the name *G. graminis* var. *tritici* var. nov. (Ggt) (Walker, 1972). Walker agreed with Turner (1940) in that the fungus causing take-all on oats should be known as *G. graminis* var. *avenae* (Gga) as it produced unlobed hyphopodia and ascospores larger than those of the other varieties.

Microscopic investigations of *Phialophora*-like isolates suggest that they are closely related to *Ggt*. When sections of *Ggt* were subcultured they resulted in conidia

producing isolates similar to *Phialophora* sp. This suggests that this species of fungi may be the asexual, anamorph of *Ggt* (Goins *et al.*, 2002).

Isolations from rotting wheat have shown the presence of other species of fungi in the same genus as the take-all fungi. *Gaeumannomyces cylindrosporus* has shorter ascospores than *G. graminis*, and was not observed in the field (Hornby *et al.*, 1977). *G. caricis* sp. nov. was found on leaf sheaths and, was particularly associated with Cyperaceae (Walker, 1980). *G. incrustans* sp. nov. was isolated from a number of species of turf grass in the United States of America (Landschoot and Jackson, 1989). This fungus produced crust-like mycelium on wheat seedlings. It was found to be similar to *G. cylindrosporus* but it had spores which were narrower and shorter that were not round but tapered. The neck of the perithecia was longer, often reaching 672  $\mu\text{m}$ , and there were no brown hyphae at the base. Sexual reproduction with a different strain of *G. graminis* (heterothallic) resulted in a *Phialophora* anamorph. *G. wongoonoo* sp. nov. was described in 2002 by Wong as a new species pathogenic to buffalo grass but not wheat and maize. This species was found to have even shorter ascospores of 36 to 75  $\mu\text{m}$ .

### 1.1.2 Infection

*Gaeumannomyces graminis* has been shown to have an affiliation with the plant debris from a previous host crops. The fungus infects the new crop of a host when it is sown. Mycelium grows from the plant debris to the roots of the seedlings infecting their roots, causing a primary infection (Bailey and Gilligan, 1999). Through the winter the fungus is able to grow on the seedlings and as the season progresses causes secondary infection in neighbouring plants by the spread of mycelium. In spring and summer the infected plants may show stunting followed by premature ripening. Blackening of the stem base occurs and perithecia may develop. After harvest the fungus survives in the plant

debris. The role in the life cycle (Figure 1.2) of any ascospores that may be released is unclear.

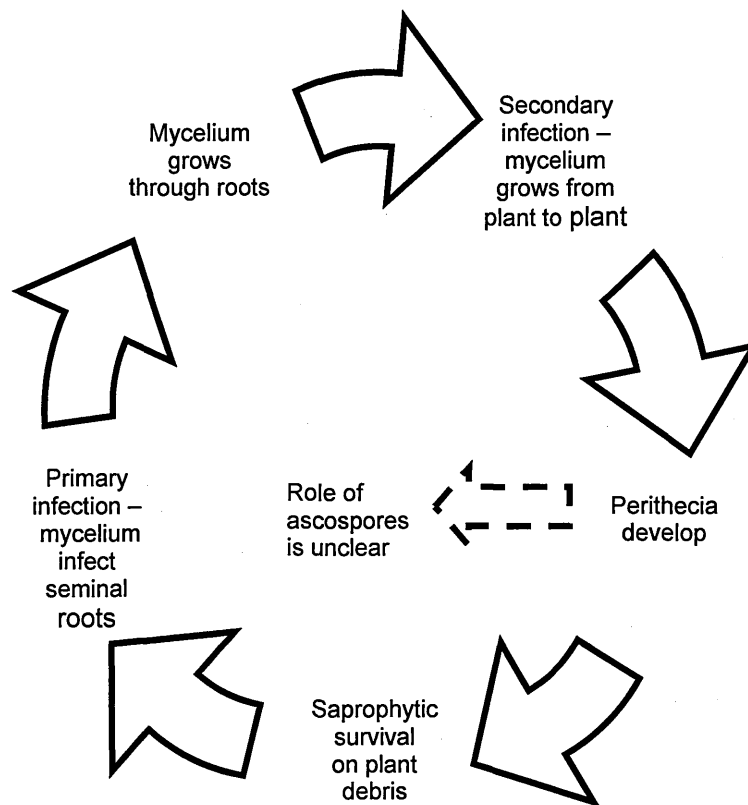


Figure 1.2: Life cycle of *Ggt* (adapted from Hornby, 1998a).

Brown and Hornby (1971) noted that *O. graminis* had been described as an ecological obligate parasite meaning that it does not grow without the presence of the living host. This is in contrast to their work which saw growth of the fungi on plant residues. Mycelium was observed growing towards wheat roots where it formed clumps of cells after five days in the presence of the host, before it entered the seminal roots. The clumps were thought to be a complex arrangement of hyphae and were similar to phialophore that the fungus produces. The authors suggested that environmental conditions determine which were produced. Production of the clumps is thought to be due to the mycelium having insufficient nutrient reserves to infect the roots. Nutrients could be

obtained from root exudates (Weste, 1972) or by the fungus breaking down surrounding plant residues. The clumps of cells do not appear to be in contact with the roots and lyse after the roots are infected by runner hyphae. Older sections of mycelium were observed as being highly branched becoming brown and granular with age. Lead hyphae, the first hyphae to come into contact with the host, often produced whorls near to the roots and swellings similar to appressorium were seen prior to infection of the roots. After six days the fungus had infected the seminal root cortex and the clumps started to lyse. Brown hyphae had started to grow over the roots by day nine with thin hyphae penetrating the roots in places. At 21 days, runner hyphae of five to seven micrometres in diameter were observed throughout the roots. These hyphae appeared to be gaining a dark brown colour and thickening with time. At this time all original clumps were reported as having lysed. Microscopic work, using light and scanning electron microscopes, by Liu *et al.* (2000) showed *Ggt* producing runner hyphae which extended along the wheat roots and their hairs. They describe the hyphae matting together which may be synonymous with the clumps that von Arx and Olivier (1972) observed. Liu *et al.* (2000) saw branching of the hyphae develop, referred to as a bridge, and simple hyphopodia were seen on the surface of the roots. Evidence suggests that after five days, growth up the roots was faster than that down the roots. Epidermal cells were penetrated by thin hyphae which accessed the cells through the intercellular spaces and the base cells of the root hairs. Penetration of the cortex occurred 12 hours later, and the cortical cells at 60 hours. Single or groups of hyphae grew through the inter and intracellular spaces. After 120 hours the hyphae reached the stele affecting the flow in the vessels. The growth of this fungus on the surface of oat roots was similar. Some hyphopodia were seen but were of a kidney or fork shape. Intercellular spaces were penetrated by hyalinated hyphae. Here the hyphae progression through the plant showed signs of inhibition, in contrast to when it infected wheat. The hyphae became coiled or swollen but were still able to spread into the cortex. Here the

hyphae were often dissolved, fewer hyphae were able to penetrate the stele and some of those that did were dissolved.

Weste (1972) observed that *O. graminis* var. *avenae* was able to infect oats and wheat. Death of the roots system was seen when the fungus had infected the xylem and blocked the vessel resulting in cell death. The fungus was seen to cause black lesions on the roots, which extended into the endodermis and sometimes covered the entire root. Observations indicated that the growth of the fungus was concentrated in the young growing parts of the roots. This was thought to represent the fungus' need for an external source of nitrogen.

### 1.1.3 Populations

A number of methods have been used to attempt to map the *Gaeumannomyces* genus. Dendrograms produced from restriction fragment length polymorphism (RFLP) studies of US isolates showed that *Ggt* and *Gga* were more closely related to each other than they were to *Ggg*. The three *G. graminis* varieties appeared to be more closely related to each other than they were to *G. incrustans*. Both of these species are more closely related to each other than they were to *G. cylindrosporus* and *G. leptosporous* (Fouly *et al.*, 1997). A phylogenetic tree of the *Gaeumannomyces* genus, using DNA amplified by the ITS 4 and 5 primer pair (including the 5.8S gene, the internal transcribed spacers, and a number of bases from the 18S and 28S genes) showed *Ggg* as being more variable than *Gga* or *Ggt*, resulting in three distinct groups within *Ggg*. The second and third groups of *Ggg* are more closely related to *Ggt* and *Gga* than to the other group of *Ggg* (Ward and Bateman, 1999). An alignment of the ITS 1, the 5.8S rRNA and ITS 2 sequences of a number *G. graminis* isolates, mainly from Germany highlighted that the *Ggt* and *Gga* are more closely related to each other than they are to *Ggg* (Ulrich *et al.*, 2000). This

supported work by Bryan *et al.* (1995) on a number isolates from Europe, the US and Australia, which demonstrated that the ITS 1 and 2 regions of *G. graminis* varieties were more similar between *Gga* and *Ggt* than either were to *Ggg*. Here the alignment indicated that the *Ggt* isolates that attack oats were more closely related to *Gga* isolates than to the other *Ggt* isolates. Interestingly, this report suggests that *Magnaporthe grisea* is more closely related to *G. graminis* than *G. incrustans* and *G. cylindrosporus* are. The work appeared to support a theory that *Phialophora graminicola* is the anamorph of *G. cylindrosporus*. Sequencing of rDNA led Ward and Bateman (1999) to the conclusion that *G. graminis* var. *maydis* is the teleomorph of *Phialophora zelicola* and *P. radiculicola*.

The country of origin of the isolates is important particularly as the inoculum spreads from plant to plant rather than by wind dispersal because populations can become isolated. Genetic drift can occur resulting in different population of *G. graminis* in different locations having significantly different DNA. This would influence any molecular test, such as PCR used to detect the species or its varieties. Work in Australia (Harvey *et al.*, 2001) indicated that genetic flow between populations is restricted and genetic drift would be a cause of any change in the DNA. The host also appeared to offer a selection pressure as DNA varied in populations associated with different crops.

#### **1.1.4 Pathogenicity**

Isolates of both *Ggt* and *Gga* have been found to produce a manganese lipoxygenase which degrades cell membranes allowing the hyphae to access the plant (Hornsten *et al.*, 2002). The pathogenicity of *Ggt* may be linked to its production of a proteolytic enzyme, polygalacturonase. An increase in polygalacturonase was correlated with an increase in the pathogenicity of *Ggt*, in contrast, *Phialophora* sp. and *P. graminicola* did not produce the enzyme and are weakly or non-pathogenic to wheat



(Martyniuk, 1988). *Ggt* has been consistently demonstrated as a pathogen of wheat (Liu *et al.*, 2000; Golldack *et al.*, 2004). It has been possible to correlate the increase in disease observed on wheat roots with the concentration of *Ggt* DNA found in those same roots using slot-blot hybridization (Herdina *et al.*, 1996).

Rye grass has been observed as susceptible to *Ggt* infection but appears to develop few symptoms (Dewan and Sivasithamparam, 1990). It has been demonstrated that *Ggt* can be split into two groups those that do not infect rye and those that do. Both groups appear to be evenly distributed within a wheat crop (Hollins and Scott, 1990). The use of restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) to determine pathogenicity determined that there were two types of *Gga* and *Ggt*. Those that attack rye (R -type) and those that do not (N - type). *Ggg* were distinguishable from *Ggt* and *Gga* isolates by these methods (Bryan *et al.*, 1999).

Litvintseva and Henson (2002a; 2002b) demonstrated the presence of three laccase genes, LAC1, LAC2 and LAC3 in *Ggt* and LAC1 and LAC2 in *Ggg*. They observed LAC3 in *Ggt* as only being active in the presence of the host, suggesting that the product of this gene may have a role in pathogenicity.

Turner (1940) suggested *Gga* as the causal agent of take-all in oats. Previous to this it was thought that this crop was resistant to the disease. It was found that the growth of *Ggt* was inhibited in the cortex and stele of oat roots, some hyphae appeared to have dissolved – the cell wall and cytoplasm were degraded (Liu *et al.*, 2000). Studies have demonstrated that the *Gga* isolates are more resistant than *Ggt* to saponins known as avenacins. *Gga* isolates were found to produce an enzyme named avenacinase (Osbourn *et al.*, 1991). *Ggt* isolates do not appear to produce this enzyme and consequently are either unable or are restricted in their ability to infect oats. A species of oats (*Avena longiglumis*) which did not appear to produce avenacin is susceptible to *Ggt*, suggesting that the presence of this saponin provides resistance to infection and the degrading of it would affect pathogenicity (Osbourn *et al.*, 1994). It would appear that the presence of avenacin

influences the colonies of fungi around oat roots. Fungi isolated from oat roots were avenacin A-1 resistant whereas those from wheat roots were either resistant or non-resistant (Carter *et al.*, 1999).

*Ggg* has been observed to cause take-all on grasses (McCarty and Lucas, 1989). Its ability to survive between hosts may be enhanced by heavily melanized cell walls. Melanin producing fungi were seen to be resistant to ultraviolet light. Melanin-producing fungi were able to grow at 30°C whereas non-melanin producing fungi did not (Frederick *et al.*, 1999).

It is possible that the pathogenicity towards a host could be used to determine the variety of an isolate of *G. graminis*. Using the size of asci to determine the variety has been seen to be less reliable than using pathogenicity. Asci produced in culture tended to be larger than those produced on host tissue. Oat-attacking isolates of *O. graminis* produced asci of 79 to 131 µm in length. Asci of the wheat-attacking isolates are 66 to 123 µm. This overlap reduced the reliability of using asci for identification (Chambers and Flentje, 1967). Isolation experiments conducted in Australia resulted in *G. graminis* fungi with longer ascospores than the average. These *Ggt* isolates attacked oats. This led the researchers to believe that there was an oat attacking subgroup of *Ggt* (Yeates, 1986).

### **1.1.5 Inoculum and Survival**

Take-all inoculum is known to survive on plant debris from the previous host crop, and occur in the soil organic matter (Shou, 1981) in the fraction 250 µm to 2 mm (Hornby, 1968; Ophel Keller *et al.*, 1995). This is the main source for an epidemic, rather than ascospores, whose role in epidemics is unclear (Hornby, 1998a). As a result various factors that influence the amount of inoculum will determine the severity of the primary infection.

Garrett (1934) reported on Australian take-all epidemics in a thirty year period, which were believed to occur only when there was high spring rainfall. Fellows (1941) demonstrated that *Ophiobolus graminis* appeared to be unaffected by sudden changes in temperatures, from those conducive to growth to freezing temperatures. These results suggested that the conditions that are most likely to produce a decline are warm (21.4 to 39.2 °C) and moist (soil was watered) whereas those least likely were cool (3 °C), moist soils which were compacted. Yield losses were positively correlated ( $r^2 = 0.91$ ) with disease incidence and increased rainfall in September (Growth Stage 24; Zadoks, 1974) in Australia (Roget and Rovira, 1991).

Size of the infection particle appears to influence the extent of the infection. As the size of artificial inoculated crowns increased so did the severity of the disease, although fragments of between 0.25 and 0.5 mm resulted in little or no disease (Wilkinson *et al.*, 1985). It has been suggested that the most infective part of the soil is zero to three centimetres. Consequently, direct drilled plots would be planted below this layer (Cotterill and Sivasithamparam, 1988a). Ploughing appears to have the effect of spreading the inoculum vertically through the soil, thus increasing the area of soil that is infective. Infectivity of the “infectious fragments”, greater than 420 µm, decreases with age. The maximum infectivity being in the weeks before harvest and just after harvest when plots were ploughed (Hornby, 1975). Break crops may also have an influence on the amount of inoculum as propagule numbers of *Ggt* were as high in wheat fields previously in pasture as those in second wheat (Cotterill and Sivasithamparam, 1988b). An interesting finding as wheat is considered the most susceptible crop to *Ggt*.

### 1.1.6 Control

#### Cultural

The control of take-all is often difficult with few chemicals making an impact. In the past it has been controlled by crop rotation and late drilling times, which have been found to reduce inoculum concentrations as well as disease severity. It has been found that a one year break from cereal can result in a 28% reduction in the extent of take-all within a field (Polley and Thomas, 1991). Planting a non-host crop causes the decline of the fungus as its nutrient source is depleted. As *Ggt* is known to infect barley, rye, oats, maize (Shou, 1981) and triticale these crops would be inappropriate to use as break crops. More severe symptoms have been observed when barley and triticale (3.2 and 10.7% whiteheads, respectively) were used as a break crop when compared to lupins and field peas (0.1 and 2.6% whiteheads, respectively). The lupins and field peas resulted in at least 229 kg ha<sup>-1</sup> more grain (Kollmorgen *et al.*, 1983). *Ggt* has been observed on grasses (Deacon, 1974) and grass pasture is thought to harbour as much inoculum as wheat (Cotterill and Sivasithamparam, 1988b). Whereas, if a field is left in fallow then the percentage disease index declines by 3.92 (Cotterill and Sivasithamparam, 1987a). A third year of wheat had 1.4 t ha<sup>-1</sup> less yield than a first year of wheat after two years of spray-topped clover (Conventry *et al.*, 1989). In second wheat crops where oilseed rape is grown as a rotational break crop can result in a slight reduction in yield (0.36 t ha<sup>-1</sup>) when compared winter beans but it is still recommended as a break crop (Hornby, 1998a).

In UK field trials Werker and Gilligan (1990) found that sowing wheat in naturally infested fields between 21 and 30 September resulted in a greater mean of disease occurrence for percentage of diseased plant (AUDPC equalled 8013 in 1984, 13381 in 1985 and 9090 in 1986) than those fields planted between the 11 and 18 October (AUDPC equalled 5681 in 1984, 7795 in 1985 and 8576 in 1986), although this difference in disease

did not always continue through the season. In two of the three years of the experiment, the percentage of diseased plants was not statistically different later in the season. The fact that the late sown plots often resulted in the same amount of disease at a later stage in the season suggests that take-all increases more rapidly in the late sown crop (Bateman and Hornby, 1999). The inconsistency of sowing date influencing the amount of take-all could be linked to other factors, such as weather, having a greater effect on disease in some years (Cotterill and Sivasithamparam, 1988c). More recent work demonstrated that a second year of wheat drilled in the same field in September reduced the amount of yield by  $0.9 \text{ t ha}^{-1}$  when compared to those drilled in October (Knight, 2002). It has been suggested that by drilling later in the year the amount of inoculum is reduced due to the degradation of its nutrient source, host plant material. In the absence of the host plant the amount of inoculum declines (Cotterill and Sivasithamparam, 1987a). As a result there is less inoculum available to infect the new crop.

The severity of disease often depends on soil conditions and climate, which can be linked to the survival of the inoculum. Wet and warm soil (10 to 40 mm of rain, below  $30^\circ\text{C}$ ) has been linked to decreases in inoculum. Dry and hot conditions (no rainfall, temperatures over  $30^\circ\text{C}$ ) in January and February (post harvest) have been linked to increases in disease (Cotterill and Sivasithamparam, 1988b). In glasshouse experiments, conducted in Australia, wetting of the soil when temperatures were in excess of  $30^\circ\text{C}$  resulted in a 70% reduction in a disease severity assay and an 80% reduction in propagule number. The disease severity was 70% higher in those soils that were not watered. Cotterill and Sivasithamparam (1987b) suggested that this is due to the wet conditions facilitating an increase in microbial activity of microorganisms other than *Ggt*.

Degradation of plant material decreases the severity of take-all. When direct drilling is used plant debris, with the fungus growing on it, remains to infect the following crop, so it would logically be expected to result in severe take-all symptoms. This is in contrast to research in Australia which found that direct drilling a wheat crop resulted in a

reduced level of disease when compared with deep cultivation, where the soil was inverted to a depth of 25 cm. Analysis of crops in the season after direct drilled ( $\text{Log}_{10}$  (mean propagule number +1) = 0.797) and deep cultivation ( $\text{Log}_{10}$  (mean propagule number +1) = 0.748) demonstrated a larger number of infective propagules than those cultivated with a rotary hoe to a depth of 6 – 7 cm ( $\text{Log}_{10}$  (mean propagule number +1) = 0.380) (Cotterill and Sivasithamparam, 1988a). Perhaps ploughing the debris into the soil increases its break down by the soil microflora, reducing its availability to the fungus. The evidence (Cotterill and Sivasithamparam, 1988a) suggests ploughing appears to spread the inoculum evenly through the soil where the seeds are planted. Direct drilling did not appear to disturb the most infective part of the soil, placing the seeds below this inoculum. This could enable the seedlings to have less disease as they have achieved some growth before coming into contact with the fungus. Although, it has been suggested that ploughing the plant debris into the soil to a depth of more than 15 cm may be beneficial in increasing the distance between host and pathogen. A greater distance between host and pathogen has been linked to reduced disease (Kabbage and Bockus, 2002). In conclusion, it is believed that there are three main cultivation effects that decrease the severity of take-all;

- if the seedling roots are separated from the inoculum
- increasing microbiological activity by mixing of the soil, and hence decreasing the crop debris
- compact seed beds decrease the ability of the mycelium to spread (Hornby, 1998a).

In analysing the growth of *Ophiobolus graminis*, Garrett (1940) determined application of nitrogen increased the activity of the fungus, although the form of nitrogen used was not detailed. Work by Garrett in 1944 went further to suggest that the application of nitrogen and dextrose solution allowed extensive growth of branched mycelium. The author thought that the planting of crops, such as mustard, that used these nutrients would reduce the amount of fungus available to infect wheat. This work is in contrast to work

completed by Glynne (1951) which showed that spraying sulphate of ammonia in the spring resulted in up to 82% reduction in the area affected by take-all, and could increase yield. These conflicting results have been linked to changes in the rhizosphere pH resulting in an environment conducive or suppressive to disease (Smiley and Cook, 1973).

Ammonium nitrate has been seen to have beneficial effects on the yield, in particular when it is applied late in a season (Schoeny *et al.*, 1998). Some results suggest that it is the severity rather than the incidence was influenced by soil pH and nitrogen source (Christensen *et al.*, 1987). The authors suggested that measurements of disease only six to eight weeks after the application of nitrogen may not reflect the grain yield at harvest. Increasing pH and the application of ammonium nitrate increased the severity of take-all by 20 to 40% (Christensen *et al.*, 1987). Application of nitrogen was positively correlated with disease incidence of take-all on seminal roots and severity of primary infection, whereas it was negatively correlated with disease on nodal roots and secondary infection cycle. The use of ammonia in contrast to ammonium nitrate resulted in a negative correlation with disease incidence (Colbach *et al.*, 1997). Werker and Gilligan (1990) observed a decrease in disease with the autumn application of ammonium chloride. It appears that the form of nitrogen that is applied to a crop and, the timing of applications is important in whether it increases or decreases take-all symptoms. Applications of ammonia or ammonium based compounds seem to be beneficial to the crop when it is actively growing.

Reis *et al.* (1982) studied the effects of other nutrients; phosphorus, potassium, magnesium, calcium, zinc, sulphur and copper. The addition of 2 H (double the concentration in Hoagland's solution) of the first three resulted in at least a 20% lower severity index score compared to calcium and sulphur. Treatments of copper and zinc were also beneficial in reducing take-all. Manganese appeared to have suppressive effects when applied to the pots. In its absence the disease severity index was greater than two, where as when it was applied it was less than two. The uptake of some of these

micronutrients appears to be effected by pH (Reis *et al.*, 1983). Zinc uptake appeared to be optimum between pH 4.5 and 5.5, whereas the uptake of copper, magnesium and iron was reduced at pH levels of between 7.5 and 8.5. Take-all symptoms decreased with the uptake of nutrients by the plants. Plant vigour appeared to increase with the application of nutrients (Reis *et al.*, 1982).

Take-all lesions appear to prevent the uptake of nutrients, by infecting the phloem. This was observed as a reduction by half of the nitrogen found in lesions of greater than one centimetre. Some plants were seen to attempt to compensate for this by increasing uptake in regions without lesions but this did not allow the plants to accumulate nitrogen content in line with healthy plants (Schoeny *et al.*, 2003).

Host resistance may influence the extent of an epidemic and as such may be used as a method of control. There is some evidence that a number of wheat cultivars are resistant to *Ggt* (Penrose, 1985). Root and cortical browning has been positively correlated with an incidence of infected seminal and coronal roots on plants in anthesis in Australian field trials. Browning appears to be the plants response to being infected with hyphae, and is thought to be due to lignification (Penrose and Neate, 1994) observed when *Ggt* enters plant cells (Liu *et al.*, 2000).

## Biocontrol and Take-all decline (TAD)

A decline in take-all has been observed in a number of monoculture experiments where there was a decline in disease symptoms or an increase in yield after a number of years of growing wheat continuously. In 1972, Shipton reported on the effect of growing wheat continuously in field trials in the UK. They observed a 25 to 90% increase in the percentage tillers per plant that were infected within a field until between three and seven years at which point there was a decrease of about 10 to 20%. Field experiments carried out by Hornby (1998b) in the UK demonstrated a 45 % decline in take-all severity in the



fifth year of wheat. A number of strains of bacteria have been associated with soils suppressive to take-all, that have had a number of crops of wheat grown on them (Barnett *et al.*, 1999). Soils in this state are said to be in take-all decline (TAD).

Bacteria have been seen to be more prevalent on the lesion of take-all infected roots compared with healthy roots. Counts of bacteria from tillering and mature plants were positively correlated with disease on test seedlings. *Pseudomonas* spp. were isolated from the lesions but failed to show a correlation with disease (Brown, 1981). *Bacillus cereus* var. *mycoides* and *B. pumilis* when applied as soil drenches in the field showed variable effects on take-all. Approximately, half the tests resulted in increases in take-all and half in decreases. Any effects did not last late in the season, disappearing by June. *Pseudomonas fluorescens* applied to the seed showed no effect on the amount of take-all (Hornby *et al.*, 1993). In laboratory experiments, aerobic bacteria including pseudomonads were significantly higher ( $5.4 (\log_{10} \text{ cfu cm}^{-1} \text{ roots})$ ) on lesions attributed to *Ggt* than on healthy roots tissue ( $3.4 (\log_{10} \text{ cfu cm}^{-1} \text{ roots})$ ) or in the soil (Barnett *et al.*, 1999). This indicates the possibility of these bacteria being linked to take-all, perhaps being antagonists to the fungus. Increased numbers of *Pseudomonas* spp. have been observed in infected roots from plants grown in naturally infested soil when compared to healthy roots. In particular, 2, 4, diacetylfloroglucinol (*phlD*<sup>+</sup>) producing strains showed inhibition towards *Ggt* (McSpadden Gardener and Weller, 2001). These authors suggested that populations of *Chryseobacterium* may enhance the ability of *phlD*<sup>+</sup> pseudomonads to inhibit *Ggt*. Field experiments completed in two years in Sweden by Amein and Weber (2002) with a *Pseudomonas* sp. strain L 18, showed a delay in disease development from October to April, although the treated plots showed no difference in disease later in the season. This early protection may be sufficient to reduce yield loss.

Changes in the populations of fungi have been observed in a sequence of wheat grown at Rothamsted, UK. Over a 100 species of fungi were isolated from the rhizosphere of wheat roots. An increase in the number of fungal species isolated was seen as more

wheat crops were grown. In 1997 a single crop of wheat resulted in 4.24 fungi identified per root piece whereas, 4.99 were identified from plants in a third crop of wheat and 5.70 in continuous wheat ( $p = 0.001$ ). *Fusarium culmorum* populations were shown to increase in the same manner, but no link was established between a particular fungus and a particular stage of TAD (Bateman and Kwasna, 1999). The diversity of fungi on the wheat roots was seen to increase when a third wheat crop was planted. The use of fluquinconazole as a seed treatment did not appear to change the fungal community, while it decreased the amount of take-all. Take-all increased in the fourth year of wheat where the third year was treated but the fourth year was untreated. This suggested to the authors that the seed treatment delayed the start of TAD by preventing the growth of *Ggt* (Dawson and Bateman, 2001; Bateman *et al.*, 2003a). It has been shown that it is necessary to have a maximum amount of inoculum to induce TAD (Shipton, 1972). Work completed by Cook (1981) suggested that TAD does not influence the initial infection but affects the subsequent progression of the disease.

## Fungicides

In 1973 a number of fungicides, including benomyl, were found to have no effect on the extent of take-all on spring wheat when applied as seed dressings or as sprays (Jenkyn and Prew, 1973). Conversely, in 1984, Bateman found that by applying Benomyl as a drench a 18 to 70% reduction in percentage of plants with take-all was observed, although this was not fully represented by yield gain. These experiments showed the fungicide, nuarimol to have some action against take-all. With both fungicides needing to be applied in high amounts and their effect on the disease not affecting the yield it was thought that they would not control the disease sufficiently to warrant their cost. A significant reduction of between 30 and 60% in the incidence of whiteheads was seen when benomyl granules and pellets were applied to the soil of naturally infested field sites. In

the same experiment imazalil granules and triadimefon (triazoles) granules and pellets resulted in a reduction of disease of over 80% when compared to the control. Ballinger and Kollmorgen (1986) suggested that triazoles apparently gave increased control of take-all due to their persistence in the soil. They suggested that the cost of the fungicides was uneconomical but by adjusting the concentration, and taking into account the scale of farm application the fungicides may become more viable as a choice in farm management. A comparison of sterol inhibiting fungicides nuarimol, imazalil, prochloraz, triadimenol, bitertanol, propiconazole, etaconazole and diniconazole in *in vitro* inhibition tests showed that they all had action against *Ggt* at high concentrations. Of the five tested in inoculated field trials – triadimenol, propiconazole, diniconazole, prochloraz and imazalil – only triadimenol was effective under high inoculum levels (Garcie and Mathre, 1987). Soil applied triadimenol was able to decrease take-all at 1 kg active ingredient per hectare in small field plots (Bateman *et al.*, 1994). The effect of nuarimol and benomyl was thought to improve with the increasing inoculum pressure (Hornby *et al.*, 1993).

Triadimenol and fuberidazole are the active ingredients in the seed treatment Baytan (Bayer CropScience Ltd., Cambridge, UK). Baytan was shown to have some action against take-all by reducing the proportion of diseased roots relative to diseased plants (Werker and Gilligan, 1990). Its action was inconsistent but it did appear to protect earlier sown crops. When compared to silthiofam (Latitude), Baytan was found to control take-all but to a lesser extent. Knight (2002) suggested that it was an alternative to Latitude when disease risk is low as silthiofam is more expensive. Application of Latitude, approved in 2001 (Monsanto UK Ltd., Cambridge, UK), has been shown to improve yields by an average of 0.46 t ha<sup>-1</sup> when second wheats were sown in September, compared to Beret Gold (fludioxonil). Latitude appears to be of particular benefit when the disease is severe. It has been demonstrated that if this fungicide is used to treat wheat sown early in the season – late September – similar yields are obtained as those achieved when seed is treated with Baytan or Beret Gold and sown October.

Fluquinconazole, applied as Jockey (Bayer CropScience Ltd., Cambridge, UK) with prochloraz, has shown similar results to Latitude in the field, in protecting the crop from yield reducing disease. It has been recommended as an application for second or third wheats as it can significantly improve the yield obtained (Bateman *et al.*, 2003b). The product name of Jockey was bought by BASF Aktiengesellschaft (Limburgerhof, Germany), but Bayer CropScience Ltd still use the name Jockey for a product consisting of only fluquinconazole (167 g l<sup>-1</sup> a.i.) in Australia. In the UK, Bayer CropScience marketed the original Jockey mixture as Galmano Plus and a fluquinconazole (167 g l<sup>-1</sup> a.i.) seed treatment as Galmano (See Table 1.1).

Table 1.1: Description of Fungicides used to treat take-all in wheat.

Commercial Name	Active Ingredients	Concentration of active ingredients	Company
Amistar	azoxystrobin	250 g l <sup>-1</sup> (23.1% w/w)	Syngenta Crop Protection UK Ltd
Baytan	triadimenol and fuberidazole	triadimenol (25% w/w); fuberidazole (3% w/w)	Bayer CropScience Ltd
Jockey	fluquinconazole and prochloraz	Fluquinconazole 167 g l <sup>-1</sup> (15.2 w/w); prochloraz 34 g l (3.2% w/w)	BASF Aktiengesellschaft
Galmano Plus	as above	as above	Bayer CropScience Ltd
Galmano	fluquinconazole	167 g l <sup>-1</sup> (15.5% w/w)	Bayer CropScience Ltd
Latitude	silthiofam	125 g l <sup>-1</sup> (11.83% w/w)	Monsanto UK Ltd

The use of a strobilurin fungicide, azoxystrobin, applied as Amistar (Syngenta Ltd., Cambridge, UK), as a foliar spray applied between January and May (at growth stages 21, 31 and 45) has been shown to increase yield. This yield increase was linked to a reduction of take-all on the roots but the method of action is unclear (Jenkyn *et al.*, 2000). It has been theorised that due to the unlikeliness of it being translocated from the leaves it must be able to access the roots from the soil, but how this would occur is unclear (Jenkyn and

Gutteridge, 2002). What is clear is that applications of Amistar, primarily used to protect plants from other diseases can reduce take-all.

## 1.2 Identification

To identify whether *G. graminis* fungi is the causal agent of symptoms observed in a crop or, to determine which variety of the fungus an isolate is by morphology requires a certain amount of skill and may not be conclusive. As a result researchers have tried to find more reliable methods, including microbiological methods and DNA techniques, to identify the species and separate the varieties. Holden and Ashby (1981) suggested that a method of distinguishing between *Phialophora radicicola*, *Ggt*, *Ggg*, *Gga* and *P. graminicola* would be to use an agar made from the leaves of oat seedlings. *Ggt* and *P. radicicola* were unable to grow on the agar. Some of the *Ggg* isolates were able to grow. All *Gga* and *P. graminicola* isolates grew across the plates. Other experiments demonstrated that it was possible to distinguish between some *Gaeumannomyces* species and *G. graminis* varieties by their growth rate on half strength potato dextrose agar (PDA). *G. cylindrosporus* isolates grew slowly, at a similar speed to *P. graminicola*. *Gga*, *Ggg* and *G. incrustans* grew quicker (Wetzel III *et al.*, 1996).

A selective medium, SMGGT3 was designed to identify *Ggt* based on the fact that this fungus produces a melanin pigment in the presence of L- $\beta$ -3, 4-dihydroxyphenylalanine (L-DOPA) (Juhnke *et al.*, 1984). This agar was thought by Duffy and Weller (1994) to be unreliable so they designed a semi-selective medium (R-PDA) which was based on *Ggt* changing the colour of rifampicin from orange to purple, in approximately 24 hours. Their comparisons of the two media suggested that R-PDA was the most effective at isolating *Ggt* from infected wheat.

Bateman *et al.* (1992) compared a number of different methods both, molecular and non-molecular. They found that in general ascospore measurement was able to confirm the

results of pathogenicity tests, even with some overlap of the range of lengths. Host specificity was a more reliable method to distinguish between the varieties but this was a lengthy procedure – often two months. Oat-leaf agar was more effective than oat-root agar but it could only be used with isolates known to be pathogenic. The quicker method of RFLP using *Bam*HI was able to determine the presence of *Ggt* and *Gga* but could not distinguish between them. The use of other enzymes was considered less reliable. They suggested the probe pMSU315 as a possible diagnostic tool. This probe was designed to identify *Ggt* isolates (Henson, 1989).

RFLP analysis using restriction enzymes *Eco*R I, and the pairs *Hind* III-*Eco*R I and *Hind* III-*Pst* I to cut 26S rRNA coding region of DNA resulted in fragments which appeared to be unique to the three varieties, *Ggg*, *Gga* and *Ggt*. Three subgroups of *Ggt* were observed and named T1, T2 and T3. T1 produced fragments of a different length than *Ggg* and *Gga*. The T2 group of *Ggt* appeared to have a similar pattern to *Gga*, and the T3 had all the fragments that *Gga* did. The T2 and T3 isolates had shown pathogenicity to wheat, oats and bentgrass (Tan *et al.*, 1994). Sequence analysis of the group I introns in the 26S rRNA genes of *G. graminis* suggested a method of distinguishing between varieties. Both *Ggt* and *Gga* demonstrated the presence of two introns, one was the same in both varieties and one was different whilst *Ggg* had no introns (Tan and Wong, 1996).

RAPD have shown to separate the three *G. graminis* varieties from each other and from *G. incrustans* and *G. cylindrosporus*. The evidence suggests that *Gga* and *Ggt* are more closely related to one another than to *Ggg*. *Ggg* showed a larger within variety difference than the others (Fouly *et al.*, 1996).

A monoclonal antibody (DD8 MAb) was found to recognise the presence of *G. graminis* varieties. The antigen it recognised was only found in live mycelia but there was some cross-reaction with *Rhizoctonia cerealis* and *R. solani*. Further investigation indicated the possibility of using the DD8 MAb with L-DOPA in an enrichment culture to identify the presence of *Ggt* (Thornton *et al.*, 1997).

### 1.2.1 *Gaeumannomyces* DNA probes

Probes have been used to establish the presence of the take-all pathogens for a number of years. A DNA probe, pMSU315, was designed from a mitochondrial fragment of *Ggt*. This American probe was able to identify *G. graminis* isolates but was not initially used with infected plants (Henson, 1989). Hybridisations of restriction fragments with this probe demonstrated that it had homology to the *Gaeumannomyces* spp. and *Phialophora* spp. tested (Henson, 1992). Later work found that it could not distinguish between the varieties *Ggt* and *Gga* (Ward and Gray, 1992). Further work, by Ward and Gray (1992), showed that the three varieties of *G. graminis* could be separated by different banding patterns when using the probe, GggMR1. This probe was hybridized to the PCR product of the universal fungal primers MS1 and MS2.

Ward and Bateman (1999) tested the probes pEG34 and pMSU315 in a Southern blot analysis. The probe pEG34 appeared to be able to distinguish between varieties of *G. graminis* isolated from a number of different cereals and did not hybridise to any non-related fungal DNA when tested. The probe pMSU315 resulted in bands that enabled differentiation between *Ggt* and *Gga* from other species of fungi. Although it was impossible to distinguish the maize isolates of *Ggt* from other soil fungi isolated from cereals. When the primers were used in the PCR they also produced expected results previously seen when amplifying isolates from wheat, barley, oats and rye. It appears therefore that these probes could not identify which varieties of *G. graminis* were present in maize.

In Australia, Herdina *et al.* (1997) used the probe pG158 and slot-blot hybridization to estimate the amount of *Ggt* in soil and correlated this with a soil bioassay. There was a positive logarithmic correlation between the disease severity (percentage of diseased

seminal roots with *Ggt* lesions) established using a soil bioassay and, the quantity of DNA extracted from soil that was artificial inoculated and naturally infested soils. The authors wrote that the assay identified DNA levels ranging from less than 0.1 ng to over 0.4 ng per 100 g of soil. Herdina and Roget (2000) also used the probe pG158 in a slot-blot hybridization assay to compare DNA levels to a soil bioassay. They found some correlation ( $R^2 = 0.63$ ) between these two assessment methods when the DNA was calculated as a proportion of the soil organic matter but this DNA did not appear to be a good indicator of the disease levels in the field. The lack of consistency in measurements seen using this molecular assay when compared to the bioassay were thought to be due to the differences between soil samples, such as soil type. This hybridization assay appeared to detect large amounts of *Ggt* DNA even when disease levels in the bioassay were low or non-existent.

### **1.2.2 Polymerase chain reaction (PCR)**

Polymerase chain reaction (PCR) is the *in vitro* process by which deoxyribonucleic acid (DNA) can be replicated many times (amplified) (Mullis and Faloona, 1987). Since Saiki *et al.* (1985) used this method for the diagnosis of sickle cell anaemia, PCR has been used in numerous assays to identify the presence of a specific disease or pathogen, including those in plant pathology. Diagnostic PCR assays used to detect the presence of plant pathogens are often preferable to other techniques of identification. This may be due to the fact that the pathogen is difficult to culture, such as *G. graminis* (Cunningham, 1981) or at present can not be cultured *in vitro*, for example, viruses. Some plant pathogens can not be identified from studying the symptoms or the symptoms may be similar for a number of different pathogens. It can also distinguish between closely related pathogens and those which occur within a complex. With traditional techniques it is often not



possible to identify the presence of pathogens before the disease occurs, for example in latent seedborne infections, and before the symptoms have started to develop (Doohan *et al.*, 1998). In all of these cases the use of diagnostic PCR would enable identification of an individual plant pathogen. This is possible because of the high specificity of the PCR assay. The specificity of a PCR assay is highly flexible and is largely determined by the primers and the anneal temperature. The primers can be designed to distinguish at any taxonomic level. PCR is also highly sensitive; in theory it is possible to detect one copy of the target DNA (Henson and French, 1993).

Certain reagents and conditions are required for a PCR reaction. Target DNA to be amplified is needed. A DNA polymerase is needed to make the copies of the target DNA. Short oligonucleotide sequences, known as primers, which are about 20 nucleotides (as a single strand) in length, are required as binding sites for the DNA polymerase. In addition nucleotides are required as the building blocks for the new copies of the target DNA, the PCR product. The PCR is completed in a number of temperature cycles, usually about thirty. Each cycle is split into three steps. The first step (denaturation) is where the double stranded DNA is heated so that it denatures and becomes single stranded DNA. In the second step (primer anneal) the mixture is cooled to a temperature that allows the oligonucleotide primers to bind to the target DNA. The third step (extension) the temperature is adjusted to allow the DNA polymerase to add nucleotides on to the bound primers (Steffan and Atlas, 1991; Annamalai *et al.*, 1995). The nucleotides that are added are complementary to those in the target DNA. These cycles are repeated increasing the amount of product exponentially as the product DNA can act as a target for the primers. This would not have been possible without the discovery of a thermostable DNA polymerase, resulting in the ability to complete repeated cycles of PCR without the continued addition of polymerase.

## Primer design

When designing primers a number of factors need to be taken into account. Primers are routinely designed as a pair, as the primers have to operate together under identical conditions. Primer length, annealing temperature and the secondary structure of the primers all has an influence on the design of primers (Henson and French, 1993). Primers are generally between 18 and 30 nucleotides long with a guanine and cytosine content of about 50%. Care must also be taken to avoid complementarity between the primers particularly at the 3' end. This is to avoid the primers binding to one another, forming primer-dimers, rather than to the target DNA. In the same way inverted repeats should be avoided to stop the primers from annealing to themselves (self-complementarity). The optimum annealing temperature of a primer pair is estimated from the primers' melting temperature and determined empirically. The optimum anneal temperature can vary depending on the PCR conditions used. Too high a temperature results in reduced amplification efficiency, resulting in reduced yield of the target DNA. Too low an anneal temperature results in reduced stringency, resulting in the amplification of non-target DNA (Steffan and Atlas, 1991; Saiki, 1992).

In primer design the sequences that are to be amplified are often pieces of DNA that occur more than once within a cell. Ribosomal genes are frequently chosen for their high copy number within a cell. Particular sequences between these genes called Internal Transcribed Spacers (ITS) have been used to develop primers to identify the presence of specific fungal pathogens. The ITS regions are known to vary mainly at the species level although there is some variation at the sub-species level (Annamalai *et al.*, 1995).

## PCR assays to detect plant pathogens

Various PCR assays have been developed to identify the presence of fungal and bacterial pathogens of plants. Parry and Nicholson (1996) used species specific primers to detect *Fusarium poae*, a causative agent of ear blight in cereals. RAPD assays were used to establish markers unique to isolates of that species. After Southern blots of isolated fragments of DNA confirmed that they were unique to *F. poae*, they were sequenced and the primer pair, Fp82 were designed and shown to be species specific. Competitive PCR assays were developed for *Microdochium nivale* var. *nivale* and *M. nivale* var. *majus* in a similar way to quantify this pathogen in wheat seedlings (Nicholson *et al.*, 1996). Later other fungi (*F. culmorum*, *F. avenaceum* and *F. graminearum*) from the Fusarium ear blight complex were detected using similar assays (Doohan *et al.*, 1998). They were quantified, along with *F. poae* and *M. nivale* varieties, in wheat ears using competitive PCR (Doohan *et al.*, 1999). PCR assays for the detection of *Rhizoctonia cerealis*, which causes sharp eyespot in wheat (Nicholson and Parry, 1996) and, *Tapesia yallundae* and *T. acuformis*, which cause cereal eyespot (Nicholson *et al.*, 1997), have also been developed to detect and quantify fungi in plant material.

Recently a method for quantifying DNA using fluorescent probes has been developed. Real-time PCR assays allow the quantification of pathogen DNA and calculation of the DNA concentration without using gel electrophoresis. This molecular assay has been developed for a number pathogens including *R. cerealis* AG-3 (Lees *et al.*, 2002) and *F. solani* f. sp. *phaseoli* (Filion *et al.*, 2003).

### *Gaeumannomyces* PCR assays

A number of authors have developed primers specifically for the use of determining the presence of *G. graminis* varieties. These include pGt1 and pGt2 by Bryan *et al.* (1995),

the KS1F and KS2R assay improved by Ward in 1995, NS5:GGA-RP and NS5:GGT-RP primer pairs (Fouly and Wilkinson, 2000) and most recently, the forward primers *Gga*, *Ggg* and *Ggt* amplified with the reverse primer AVE/R (Rachdawong *et al.*, 2002). These primer pairs have been used diagnostically to test whether a fungal isolate is a *G. graminis* variety but few have been used to determine the presence of take-all within plant samples obtained from the field. As far as this author is aware the pGt1 and pGt2 is the only primer pair that has been used to quantify *Ggt* in roots using competitive PCR although the only evidence for this is a figure in a book (Hornby, 1998a).

### **1.3 The aims of this thesis were;**

- To develop DNA extraction procedures for plant material infected with take-all pathogens of wheat
- To identify a PCR assay capable of distinguishing the take-all pathogens of wheat
- To develop a competitive PCR assay to quantify the take-all pathogens of wheat
- To test the ability of competitive PCR assay to quantify take-all pathogens of wheat in commercial wheat fields.
- To determine if the severity of take-all in wheat can be predicted by quantifying inoculum of take-all pathogens using a competitive PCR assay
- To determine if take-all pathogens of wheat can be monitored through the growing season and to measure the efficacy of seed treatments towards take-all using a competitive PCR assay.

## **Chapter Two**

### **General Materials and Methods**

# General Materials and Method

All chemicals were supplied by Sigma, UK unless otherwise stated.

## 2.1 Isolates

All isolates were stored on potato dextrose agar (PDA; Merck, Darmstadt, Germany) slopes at 4 °C. A list of isolates and their source is provided in Appendix 1.

## 2.2 Isolation

To obtain field isolates, stubble was collected by walking a 'W' across fields that demonstrated take-all symptoms earlier in the season. Roots from infected stubble were removed from their stem bases, sterilized in sodium hypochlorite solution (1% available chlorine) for 1 min, twice washed in sterile (121 °C, 20 min) distilled water and dried using sterile filter paper. One centimetre lengths of root were placed on PDA supplemented with streptomycin sulphate (130 µg ml<sup>-1</sup>). The plates were incubated at 20 °C for 1 week on the laboratory bench and any *Gaeumannomyces graminis* var. *tritici* (Ggt) – like growth, defined as dark, highly disorganised mycelium (Figures 2.1 and 2.2), was sub-cultured onto PDA plates. One week old mycelium was removed from the plates for DNA extraction. For long term storage isolates were kept on PDA slopes refrigerated at 4 °C.

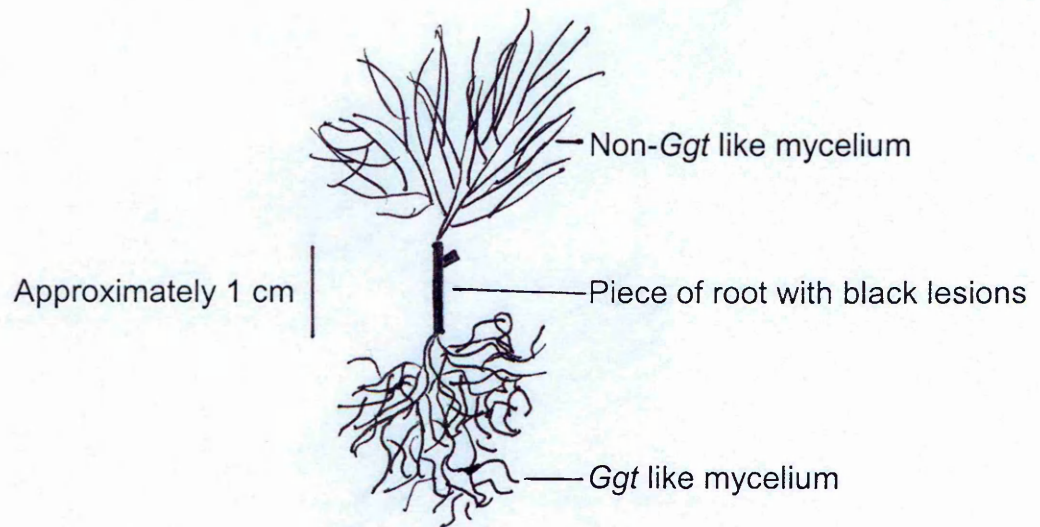


Figure 2.1: Diagrammatic representation showing *Ggt*-like and non-*Ggt* like mycelium growing from a root section.



Figure 2.2: *Gaeumannomyces graminis* var. *tritici* mycelium from PDA plates after four days at 20 °C (x 7.5 magnification).

## 2.3 Assessment of plant material

Plants were assessed for visual symptoms by washing the roots and visually inspecting them against a white background. The percentage of roots covered with black lesions was estimated. For the purpose of statistical analysis the percentage of roots covered with blackened lesions was grouped into five categories (0=0, 1 = 1 – 25%, 2 = 26 – 50 %, 3 = 51 – 75 %, 4 = 76 – 100%) for stubble plant material, six for GS 30, 39 and 69 (0=0, 1 = less than 10%, 2 = 11 – 25%, 3 = 26 – 50 %, 4 = 51 – 75 %, 5 = 76 – 100%). Stems were assessed for black lesions using a scoring system where 0 means that no disease was observed, 1 = slight disease, 2 = moderate amount of disease, 3 = severe disease and 4 means that the stem has been completely destroyed. A take-all rating, which was adapted from Bateman *et al.* (2003a), was calculated for roots and stems of samples by multiplying the amount of plants in each score category by the score for that category and adding it to the other categories. This value was divided by four in the case of the stubble samples, five for GS 30, 39 and 69. The values were then divided by the total number of plants assessed. By multiplying this value by 100 a take-all rating (TAR) was obtained with a maximum of 100. Equation 2.1 shows the calculation for the stubble TAR.

$$\frac{(1 \times a) + (2 \times b) + (3 \times c) + (4 \times d) / 4}{n} \times 100 \quad \text{Equation 2.1}$$

Where a = number of plants with 1 – 25 % of their roots covered by lesions, b = 26 – 50 % covered by lesions, c = 51 – 75 % covered by lesions and d = 76 – 100 % covered, n is the total number of plants assessed.



## 2.4 DNA extraction

A number of different methods were employed for the extraction of DNA from different sources.

### Rapid fungal extraction

Mycelium was removed from the surface of agar plates and placed in sterile 1.9-ml microcentrifuge tubes. Fifty microlitres of sterile water was added and the mycelium was crushed using a micropestle. One hundred microlitres of Chelex carbon buffer (1 g of chelex and 0.25 g carbon in 20 ml distilled water) was added to the mycelium and the tubes were incubated at 56 °C for 20 min. The tubes were vortexed and boiled for eight minutes. After cooling the tubes were centrifuged (Biofuge 13, Heraeus) at 12,000 g for 15 min. Fifty microlitres of the supernatant was added to 50 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4 °C in the short term, -20 °C for long term.

### Fungal mycelium from broth cultures

A plug of fungi from the edge of a colony growing on PDA plates was added to 250 ml flasks with 50 ml of potato dextrose broth (Difco, La Pont de Claix, France) and incubated for two weeks at 20 °C, 100 rpm.

After incubation, the broth and mycelium were transferred to a 50-ml centrifuge tube and centrifuged for 5 min at 1800 g. The broth was poured off and 50 ml sterile distilled water (SDW) was used to wash the mycelium. Centrifugation was repeated and the water poured away. The resulting mycelium pellets were freeze-dried and powdered by

placing three ball bearings (8 mm diameter) in the tubes and shaking violently. The DNA extraction was similar to the method used for plants by Edwards *et al.* (2001). Thirty millilitres of cetyltrimethylammonium bromide (CTAB) extraction buffer (sorbitol, 23 g; N-lauryl sarcosine, 10 g; CTAB, 8 g; sodium chloride 87.7 g; ethylenediamine tetraacetic acid (EDTA), 8 g; polyvinylpyrrolidone 10 g; water to 1 litre) was added to the powdered mycelium. After incubation at 65 °C for 1 hour, 10 ml of potassium acetate (5M) was added and the tubes were frozen. Thawed samples were centrifuged at 3000 g for 15 min. The supernatant was poured into a 50-ml centrifuge tube and 10 ml of chloroform was added. Tubes were mixed for 1 min and centrifuged at 1800 g for 15 min. As much of the aqueous phase was removed into a sterile tube as possible without disturbing the interface. To this an equal volume of isopropanol was added, mixed for 1 min and incubated at 18 °C for 30 min. Following incubation the samples were centrifuged at 1800 g for 15 min to pellet the DNA. Pellets were washed in 44% isopropanol and left to air dry. Dry DNA pellets were resuspended in 2 ml of TE buffer and incubated at 65 °C for 1 hour. After incubation the samples were vortexed and 1 ml aliquoted into 1.5-ml tubes, which were centrifuged at 12,000 g for 5 min. Samples were stored as for the other fungal DNA samples.

## Plant material

For each plant sample the roots were removed from their stems and placed in 50-ml sample tubes. Four centimetres of the stem bases were removed from the same plants and chopped in to one centimetre lengths. The stem bases were placed in 50-ml sample tubes. The plant material was freeze dried and milled in these tubes. Milling was carried out by shaking, with sterile steel ball bearings (1 of 22 mm diameter and 3 of 8 mm diameter) in the tubes, in a soil mill (Griffin, UK) for 30 min. The resulting powder was transferred to a

50-ml centrifuge tube and 30 ml of CTAB extraction buffer was added, samples were mixed. The samples were incubated in a water bath at 65 °C for 1 hour. After cooling 10 ml potassium acetate (5M) was added to the samples, which were mixed, and frozen for 1 hour. Thawed samples were mixed then centrifuged at 1800 g for 15 min. Following this, 1.2 ml of the supernatant was added to 0.6 ml of chloroform in a 1.9-ml tube, mixed for 1 min, and centrifuged at 12,000 g for 15 min. One millilitre of the supernatant was added to 0.8 ml of pure isopropanol. Samples were mixed for 1 min, and left at 18 °C for 30 min. Samples were centrifuged at 6000 g for 15 min and the supernatant was poured away. DNA pellets were washed with 0.8 ml of 44% isopropanol followed by 15 min centrifugation at 12,000 g. Any supernatant was removed and the washing step repeated before the DNA pellets were air dried. When the pellets were completely dry, DNA was resuspended with TE buffer at 65 °C for 1 hour. The samples were then vortexed and centrifuged at 12,000 g for 5 min. For storage samples were kept at 4 °C, or –20 °C for longer term.

## **2.5 Establishing the purity and concentration of DNA**

DNA extracted from all samples was assessed for its purity and concentration using a scanning spectrophotometer. A ten-fold dilution of the samples was made by adding 20 µl of the samples to 180 µl of TE buffer. A spectrophotometer (Beckman Instruments Inc., Fullerton, USA) was used to measure absorbance at 260, 280, 328 and 360 nm.

The concentration of DNA was calculated using the Warburg-Christian coefficient, Equation 2.2:

$$[\text{Nucleic acid ng } \mu\text{l}^{-1}] = (-36 * (\text{Abs}_{280} - \text{Abs}_{328})) + ((62.9 * (\text{Abs}_{260} - \text{Abs}_{328})).$$

The purity was calculated using the Equation 2.3:

$$\text{Purity ratio} = \frac{(Abs_{260} - Abs_{320})}{(Abs_{280} - Abs_{328})} \quad \text{Equation 2.3}$$

Pure DNA has the ratio of 1.8.

All of the DNA (except from the rapid fungal extractions) was diluted in TE buffer to the required concentration using Equation 2.4:

$$\text{Desired DNA concentration} = \frac{(D \times V)}{C} \times \left( \frac{V}{V - \left( \frac{D \times V}{C} \right)} \right) \quad \text{Equation 2.4}$$

Where, D = the desired final concentration of the DNA, V = volume of TE and C = concentration of DNA from Warburg – Christian coefficient.

In addition all dilutions were tested whether they contained DNA of sufficient purity to be amplified by the PCR. This was done by amplifying the DNA with universal primers ITS 4 and ITS 5 (White *et al.*, 1990). For this reaction, five µl of sample and 20 µl of the reaction mix were added to the reaction wells. The PCR reaction contained 100 µM of each nucleotide (AB gene, UK), 100 nM of each of the primers, ITS 4 and ITS 5, 0.5 units of Taq polymerase (AB gene), 10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 100 µg/ml gelatin and 0.05% of Tween 20 and Nonidet P-40, 5% glycerol and 40 µg/ml cresol red. Once the reaction mix has been added to the wells a drop of mineral oil was added to each well. The wells are placed in a programmable thermal cycler, PTC 100 (MJ Research Inc., Watertown, MA, USA.) for 75 seconds at 94 °C followed by 35 cycles of 15

seconds at 94 °C, 15 seconds at 50 °C and then 45 seconds at 72 °C. Once the 35 cycles had been completed a final extension period of 4 minutes 15 seconds at 72 °C was carried out. The samples were electrophoresed on a 2% agarose gel containing 0.05% ethidium bromide. The gels were visualized and recorded using a GelDoc1000 image analysis system (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK.).

## **Chapter Three**

# **Evaluation of different DNA extraction techniques**

### 3.1 Introduction

It is necessary to obtain pure DNA for use in PCR as the presence of some chemicals such as humic acids, can result in bands that are weak or non-existent on an agarose gel (Tsai and Olson, 1992a). The extraction of pure DNA from soil for use in PCR has been achieved by using various techniques that resulted in the DNA being separated from other cell components and environmental chemicals.

A brown substance has been linked to problems with the amplification of DNA using PCR when attempting to extract DNA from soil. This brown substance is thought to be humic acid. It is believed that humic acids are extracted with the DNA (Ogram *et al.*, 1987) and have been found to affect the efficiency of the PCR (Tsai and Olson, 1992a). How the humic acids effect the PCR is unclear but humic acid can inhibit amplification at concentrations as low as 10 ng per reaction volume. Tsai and Olson (1992b) thought that the inhibition might be due to the fact that these acids chelate magnesium ions. Although in further tests using high concentrations of these ions and low concentrations of a humic acid the inhibition was not overcome. Herdina *et al.* (1997) suggested that the use of an acidic, high salt buffer when extracting the DNA helps in the removal of these humic acid compounds. The authors suggested that soil type, with varying soil organic matter content, may effect the estimations of fungal content of the soil.

To overcome interference of these soil components in PCR assays DNA-binding resins have been used to extract DNA (Towner, 2000; Faggian *et al.*, 2001). DNA-binding resins bind to the DNA, which is in turn caught on a membrane while other components of a soil solution do not. A large number of papers have tested a variety of other methods including using chemicals such as polyvinylpolypyrrolidone (PVPP), cetyltrimethylammonium bromide (CTAB) (Griffiths *et al.*, 2000), sodium dodecyl sulphate (SDS) and guanidinium thiocyanate (Miller *et al.*, 1999) in an attempt to extract

pure DNA. Other authors have tried methods that introduce beads to the soil in a bead beater to rupture the cells, and some have gone on to further purify the DNA.

A commercial available kit, UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), uses beads to disrupt the cells and guanidine thiocyanate and SDS to extract the DNA. A spin filter was used to capture the DNA, which was washed with a solution of ammonium acetate, guanidinium thiocyanate and TE buffer. Towner (2000) discussed a similar methodology involving the use of guanidinium thiocyanate to dissolve cells leaving the DNA undamaged. The solution of guanidinium thiocyanate and sample was added to diatomaceous earth and placed on a filter. The DNA is separated from the rest of the cellular components as it remains on the filter while the other cell components are washed away with ethanol.

Braid *et al.* (2003) used PVPP and CTAB to extract soil DNA but they believed them to be inadequate in removing PCR inhibitors. The authors felt that chromatography methods seemed to be more effective although time consuming and expensive. Consequently they studied the addition of multivalent cations, magnesium chloride ( $\text{MgCl}_2$ ), calcium chloride ( $\text{CaCl}_2$ ), aluminium ammonium sulfate ( $\text{AlNH}_4(\text{SO}_4)_2$ ) and ferric chloride ( $\text{FeCl}_3$ ), to samples during lysis and precipitation stages of the UltraClean™ Soil DNA Isolation Kit. Both  $\text{MgCl}_2$  and  $\text{CaCl}_2$  when added at the lysis and precipitation steps resulted in DNA yields similar to the control sample, when the chemicals were not added. The addition of  $\text{AlNH}_4(\text{SO}_4)_2$  and  $\text{FeCl}_3$  at the lysis stage resulted in reduced yield but when the PCR products were analysed amplification was improved. It was found that  $\text{AlNH}_4(\text{SO}_4)_2$  when added at a concentration of 100 mM appeared to produce the strongest bands with PCR amplification. The authors hypothesised that the cations interacted with humic acids because they have an open structure whereas the DNA has a helical structure, which prevented it from being removed from the solution. They suggested that optimisation of the concentration of the  $\text{AlNH}_4(\text{SO}_4)_2$  may be needed with different soils.



Miller *et al.* (1999) compared various extraction and purification procedures to find the optimum conditions of extracting DNA from soil. They tested a phosphate buffer ( $\text{NaH}_2\text{PO}_4$ ) which was combined with SDS, NaCl and Tris. These chemicals were further tested with phenol and chloroform-isoamyl alcohol, and with Chelex 100. The various chemical combinations were added to the samples prior to them being subjected to bead beating or freeze thawing. Additional treatments using guanidinium isothiocyanate or adding a lysozyme mix (containing lysozyme, NaCl, and EDTA) with SDS prior to the bead beating and the freeze thawing were also tested. They found that guanidinium isothiocyanate was the only chemical whose addition resulted in no bands on the agarose gel. Their results indicated that the use of an organic solvent (phenol or chloroform) with SDS prior to bead milling produced a greater yield than the use of Chelex 100, freeze-thawing with SDS and the use of enzymes. Following the extraction tests these authors used the DNA they had extracted using Chelex to compare methods to further purify DNA. The four methods that were tested included were the use of Spinbind columns, agarose gel electrophoresis followed by the use of the Spinbind columns, a method using ammonium acetate, ethanol and glycogen, and a method using gel filtration (Sephadex G-200). A PCR inhibition assay was used to determine whether the PCR inhibitors had been removed. It appeared that Sephadex G-200 was the most effective at purifying the DNA, although they required a greater amount of time to prepare. The authors suggested that further purification may be necessary, as inhibitors were still present. They also suggested that freeze drying of the samples may be needed if they are to be stored prior to extraction.

Miller (2001) evaluated the use of various gel filtration methods to remove the humic acids from samples of DNA extracted from soil. DNA was extracted from the soil by a chemical lysis method or by bead mill homogenisation and purified using a number of resins, including Sepharose and Sephadex, of various bead sizes. By measuring the amount of DNA and humic acids eluted from the resins the results appeared to demonstrate that

DNA was separated from humic acids more completely if Sepharose resins were used. The author suggested the use of gravity-flow columns rather than spin columns as they are easier to set-up, being less labour intensive, and appeared less likely to become clogged. He also suggested that the gravity-flow columns may be easier to use when processing large numbers of samples. Miller concluded that Sepharose 2B produced the best results with the soil and sediments he tested. The best gel filtration resins being those that have a greater size fractionation range than humic acids but are lower than the DNA fragments.

Stach *et al.* (2001) compared the ability of the UltraClean soil DNA isolation kit, the Bathe method, a modified method adapted from Miller *et al.* (1999), a modified method adapted from Griffiths *et al.* (2000) and a modified method adapted from Zhou *et al.* (1996) to extract DNA from soil. The Bathe method involved the addition of a buffer (containing Tris-HCl, NaCl, CTAB and PVPP) and the sample to a tube containing glass beads. A bead beater was used to homogenise the samples. Following an incubation period and further bead beating the DNA was collected by centrifugation. The Miller method was similar to the Bathe method except the extraction buffer contained  $\text{NaH}_2\text{PO}_4$ , NaCl, Tris-HCl and SDS. The Griffiths buffer consisted of CTAB (NaCl,  $\text{K}_2\text{HPO}_4$ ) and phenol/chloroform/ isoamyl alcohol. The Zhou method used an extraction buffer (Tris-HCl, EDTA,  $\text{NaH}_2\text{PO}_4$ , NaCl, and CTAB) and proteinase K with SDS being added later. After the samples had been incubated they were centrifuged and the supernatant was collected. For all of the methods, once the supernatant had been collected phenol/chloroform/isoamyl alcohol, chloroform/isoamyl alcohol and isopropanol were used to isolate the DNA. The authors used agarose purification, spin-column chromatography (Sepharose CL-4B), the Wizard DNA minicolumn and a modified method obtained by personal communication from Mendum (1999) (using Sephadex G-100 and PVPP) to purify the DNA after extraction. These authors suggested that higher bead beating speeds result in increased shearing of the DNA – prevention of shearing was

important to them. DNA of high molecular mass was obtained when heat was used in the extraction protocol. They found that extraction buffers that contained SDS increased the yield of DNA but also increased the amount of co-extracted humic acids. PCR products were obtained from DNA extracted using the UltraClean kit without any further purification. The Bathe, Griffiths, Miller and Zhou methods produced PCR bands when the extracted DNA was diluted. From the purity ratios (obtained by dividing absorbance at 260 nm by that at 280 nm) it appeared that the DNA from all methods of extraction had significant humic acid contamination. The addition of bovine serum albumin (BSA) to the PCR reaction showed that when the non-acetylated form was used PCR products could be obtained with higher concentrations of humic acid. These authors found that the purification protocols they tested removed the humic acids – demonstrated by a colour change of the solutions from darker brown to yellow or clear. When the DNA was amplified by PCR after purification they all produced bands.

Lees *et al.* (2002) used a 'SPCB' buffer consisting of sodium phosphate, CTAB and NaCl, sonication and shaking using glass and zirconia beads to extract the DNA. Chloroform, sodium acetate and isopropanol were added to separate the DNA from other cell components. These authors found that they had variable results in detecting *R. solani* AG-3 in soil using a real-time PCR assay and believed that this may be due to the presence of PCR inhibitors within the soil. They felt that the use of commercial extraction kits might improve the quality of the DNA extracted.

Heinz and Platt (2000) compared two methods of extraction with the view of using them in large-scale sampling. They tested an extraction technique discussed in Volossiuk *et al.* (1995) and Heinz *et al.* (1998) that used a SDS extraction buffer and phenol. This extraction technique was compared to a procedure where the samples were added to skimmed milk powder and an extraction buffer, which contained Tris-HCl, EDTA, SDS and proteinase K. Ammonium acetate was added to remove the cell debris. Ethanol was

used to separate the DNA. In this comparison the authors found that the DNA extracted with proteinase K appeared to demonstrate the least amount of inhibition when amplified using PCR. They also suggested that samples from clay soil tend to result in DNA that is inhibited more than DNA extracted from other soils.

A number of papers describe the use of extraction methods with the intention of identifying the presence of the take-all pathogen. Ophel Keller *et al.* (1995) and Herdina *et al.* (1996) started by separating the soil organic matter from the rest of the soil by a floatation sieving method (Neate, 1987), the causative agent of take-all is found in this part the soil (Shou, 1981). This involved washing the soil through two sets of sieves (Ophel Keller *et al.*; 2 and 0.25 mm: Herdina *et al.*; 1.4 and 0.35 mm). The fraction on the smaller sieve was collected for DNA extraction. Both papers used the Raeder and Broda (1985) protocol to extract the DNA from the soil organic matter. Herdina *et al.* adapted the protocol as described below. Soil organic matter was ground in liquid nitrogen before being added to the extraction buffer (containing Tris HCl, NaCl, EDTA and SDS). Phenol/chloroform and RNase were added to the samples and incubated, in separate steps, prior to chloroform/isoamyl alcohol being used. Cold isopropanol was used to precipitate the DNA. Once the DNA had been pelleted, washed with ethanol and dried it was suspended in TE-NaCl. Sepharose CL-6B columns were used to purify the DNA (Whisson *et al.*, 1995). These authors estimated that they extracted between 70-80% of the *Ggt* DNA when using this technique with roots and suggested that it was only slightly less when the DNA was extracted from soil organic matter. They thought that some of the DNA was lost in the Sepharose column. In these experiments a slot-blot hybridization assay was found to be effective in identifying the presence of *Ggt* in soil organic matter. Ophel Keller *et al.* (1995) used their extracted DNA in a nested PCR assay. They found in their early experiments that there were inhibitors present within the extracted liquid, such that dilution was necessary to achieve positive results. To assess the inhibition within the samples

universal primers were used in amplifications of dilutions of the samples. This was used to determine the presence of false negatives. In addition the authors suggested that the soil fraction between 2 mm and 0.25 mm was the most consistent in obtaining positive results with the assay. This would indicate that the *Ggt* is predominantly located in this fraction of the soil. Herdina *et al.* (1997) furthered this work, demonstrating that *Ggt* was mainly located in fractions larger than 0.5 mm at a depth of 5 cm.

In 1997, Herdina *et al.* extracted DNA from soil organic matter in a similar way to Herdina *et al.* (1996) except the initial step of RNase and chloroform/isoamyl addition where excluded. The DNA was precipitated with cold isopropanol and ammonium acetate, washed with ethanol and suspended in TE containing RNase. The DNA was purified using chloroform/isoamyl alcohol and another addition of the extraction buffer. Cold isopropanol was used to precipitate the DNA, which was suspended in TE buffer. The Sepharose purification was not used. The authors believe that the use of a high salt buffer removed the humic acid without the need for further purification, and consequently the samples were processed more quickly, and cheaply. They appeared to have no problems with humic acids interfering with the slot-blot hybridization assay.

All of the extraction techniques described above are designed to remove chemicals that inhibit the amplification or hybridization of DNA. Attempts have been made to determine the presence of the chemicals and to quantify the amount of inhibition. Two main methods have been described – spectrophotometry and the use of an inhibition assay.

Steffan *et al.* (1988) used spectrophotometry to compare different extraction techniques. They found that absorbance ratios  $A_{260/280}$  and  $A_{260/230}$  of pure DNA were 1.8 and 2.1. A range of ratio values (from 0.8 to 2.2 for both ratios) were obtained for DNA contaminated with humic acids. The authors felt that samples with the higher values contained DNA of sufficient quality to be used in their hybridization assay although some of the restriction enzymes were inhibited (for example, *SalI*). Stach *et al.* (2001) used

absorbance ratios to determine the presence of proteins and humic acids. With the  $OD_{260/280}$  ratios of less than 1.7 indicating protein contamination and  $OD_{260/230}$  ratios less than 2.0 suggesting that there is humic acid contamination. Kuske *et al.* (1998) used PicoGreen dye in a fluorescent assay for luminescence spectrophotometry to determine the concentration of humic acid co-extracted with the DNA. They measured between 49 and 2200  $\mu\text{g}$  of humic acid extracted per gram of wet soil. Howeler *et al.* (2003) utilised a PicoGreen assay to quantify the presence of humic acids. The reproducible results they obtained were able to measure concentrations of the humic acids from 0.1 to 100  $\text{ng}/\mu\text{l}$ .

The other main method that has been used to establish the amount of inhibition present in a DNA sample is a PCR inhibition assay. These assays are designed to amplify DNA which is added to the soil DNA samples. This DNA must not normally be found in the soil as this would interfere with the results. Miller *et al.* (1999, 2001) designed an assay which amplified the 16S rDNA gene of *Methylobacterium albus* BG8. They added purified DNA to the PCR reaction with the soil DNA and subjected it to PCR amplification. The samples were scored according to whether the expected band was visualised on agarose gel. Burgmann *et al.* (2001) went further by using image analysis to quantify bands produced by a cloned *Pseudomonas* sp.

To use DNA extracted from soil in a diagnostic or quantitative PCR test to determine the presence of *Ggt* in a field it is necessary to have an effective method of extracting the DNA from large numbers of samples. Any inhibition present should not appear to effect the amplification of the product. A number of methods have been described with kits such as the UltraClean<sup>TM</sup> Soil DNA Isolation Kit (Stach *et al.*, 2000), and Towner (2000) method being suggested. Using a bead beater is thought to improve the quality of the DNA (Miller, 2001). Some authors (Griffiths *et al.*, 2000; Lees *et al.*, 2002) have used CTAB. Consequently it was felt appropriate to compare the CTAB method described in Chapter Two with the commercial kit and various adaptations of the Towner

(2000) method (including use of a bead beater) to find the most effective way of obtaining DNA from soil organic matter that amplifies using PCR. In order to determine the quality of DNA extracted from soil organic matter, spectrometry and a PCR inhibition assay were used.

### **3.2 Aims**

- Test the ability of a range of procedures to extract *Ggt* DNA from soil organic matter.
- Develop a PCR inhibition assay to assess the ability of extraction procedures to remove inhibitory compounds from DNA extracted from soil organic matter.

### **3.3 Materials and Methods**

#### **3.3.1 Soil organic matter extraction**

Soil was collected from random locations within a field (SK645011) in Stoughton, Leicestershire, using a 30 cm soil corer (6 cm in diameter). Approximately 1.5 kg of soil was placed in cloth bags and put in a ventilated drying cupboard (24°C, 1 week). Dry soil was washed through a 2 mm sieve and organic matter collected through a Fenwick can (Neate, 1987) on to a 0.5 mm sieve. The soil organic matter was transferred from the 0.5 mm sieve to muslin and dried over night at 24°C, then freeze-dried. Dried soil organic matter was milled for 20 min in a soil mill with three stainless steel balls (8 mm in diameter) in the tubes, in preparation for DNA extraction.

#### **3.3.2 DNA Extraction**

Three published DNA extraction methods were tested including the previously described CTAB method (see Chapter 2), the UltraClean™ Soil DNA Isolation Kit (Mobio Laboratories Inc., Carlsbad, CA, USA) and variations on a method by Towner (2000) using a DNA binding resin and a glass filter. Ten replicates of each method were extracted from the Stoughton soil organic matter.

##### **Extraction One – Variations on the Towner (2000) method**

Centrifugation was used to briefly spin (12,000 g) the samples instead of using a vacuum pump. For all these extractions spin filters were prepared by placing glass



microfibre filter discs (G-F/C 90 mm diameter; Whatman, Maidstone, UK.) on the filter (0.2  $\mu$ m SpinX; Costar, New York, USA) which in turn was placed in a 1.9-ml microcentrifuge tube. A solubilization buffer was made by dissolving 472 g of guanidinium thiocyanate in 500 ml of water. Fifty millilitres of 1 M Tris-HCl (pH 7.5), and 40 ml of 0.5 M EDTA (pH 8.0) was added. The solution was made up to 1 l and stored in a light-proof container at 4°C. DNA binding resin was prepared by suspending 10 g of diatomaceous earth in 2 l of water and allowing it to settle for one hour. The liquid was poured away and the remaining earth re-suspended as before. Once the supernatant was poured away, solubilization buffer made up to 900 ml was used to resuspend the earth. The solution was made up to one litre with water and stored at 4°C in a light-proof container.

The basic method (method 1A) of extraction was to add 100 mg of soil organic matter to 1.8 ml of solubilization buffer and inverted to mix. Five hundred microlitres of DNA binding resin was added and inverted to mix. The solution was pipetted on to the glass filter, 0.5 ml at a time and centrifuged at 12,000 g until all the liquid has passed through the filter, making sure that the filter paper did not dry out. Five hundred microlitres of solubilization buffer was added to the filter and centrifuged through. Next, 2 ml of ethanol wash (50 mM Tris-HCl, pH 7.5; 10 mM EDTA, pH 8.0; 0.2 M NaCl; 50% (v/v) ethanol) was centrifuged through the filter. The filter was dried to remove all the ethanol wash by spinning (12,000 g, 2 min). To free the DNA from the filter, 50  $\mu$ l sterile distilled water was pipetted on to the filter which was centrifuged at 12,000 g for 2 min. DNA was stored at 4°C.

A number of variations on the basic method (1A) were tested;

1B; as 1A except an additional 200  $\mu$ l of solubilization buffer was used to suspend the soil organic matter.

1C; as 1A except prior to adding the DNA binding resin the samples were vortexed, centrifuged (12,000 g, 2 min) and the supernatant transferred to another microcentrifuge tube without disturbing the pellet. The DNA binding resin was added to this supernatant and the extraction was continued as above.

1D; as 1C except the soil organic matter was added to a 2-ml microcentrifuge tube containing 0.2 g zirconia beads (0.5 mm diameter) and 0.2 g glass beads (1.5 – 2.5 mm diameter) and placed in a bead beater (Disruptor Genie; Scientific Industries, New York, USA) for 5 min. Solubilization buffer (1.8 ml) was added to the soil organic matter.

1E; as 1D except that 0.9 ml of the solubilization buffer was added to the soil organic matter before and after bead beating.

1F; as 1E except that all the buffer was added prior to bead beating.

1I; as 1E except the soil organic matter was bead beaten prior to 0.9 ml of solubilization buffer being added, then the tubes was put in the beater for another five minutes, then 0.9 ml of the solubilization buffer was added and the method continued as before.

1J; as 1I except that all 1.8 ml of solubilization buffer was added before the second bead beating.

1K; as 1I except that 1.3 ml of solubilization buffer was added for the second beating, 0.5 ml being added afterwards.

## Extraction Two – Mo Bio's UltraClean™ Soil DNA Isolation kit.

The method followed was as in the manufacture's instructions for maximum yields. Briefly, 100 mg of soil organic matter was added to the Bead Solution tubes and vortexed. Sixty microlitres of Solution 1 was placed in the tubes, which were vortexed prior to the addition of 200 µl of an Inhibitor Removal Solution. The tubes were vortexed horizontally for 10 mins at maximum speed, followed by centrifugation. (10,000 g, 30 secs). The supernatant was transferred to a clean 1.9 ml-microcentrifuge tube and 250 µl of Solution 2 was added. The tubes were vortexed and incubated at 4°C for 5 mins, centrifuged (10,000 g, 1 min), and the supernatant transferred to a clean 1.9-ml microcentrifuge tube. Solution 3 (1.3 ml) was placed in the tube and vortexed. The contents of the tubes were pipetted on to spin filters (no more than 700 µl at a time) and centrifuged (10,000 g, 1 min). Three hundred microlitres of Solution 4 was added to the filter and centrifuged (10,000 g) twice, once for 30 secs, once for 1 min. Next the spin filter was placed in a clean microcentrifuge tube, 50 µl of Solution 5 was added. Tubes were centrifuged (10,000 g, 30 secs) to release the DNA. DNA was stored at 4°C.

## Extraction Three – CTAB extraction

This procedure is similar to the one used for plant material, except that 1 ml of CTAB buffer was added to 100 mg of soil organic matter. Three hundred microlitres of potassium acetate, 0.5 ml chloroform and 0.8 ml of isopropanol volumes were subsequently used.

### 3.3.3 Inhibition assessment

Spectrophotometry was used, as described in Chapter 2, and an inhibition assay was designed to establish the inhibition present in the DNA samples extracted above. Spectrophotometry was used as it is the standard way to assess the quality and quantity of DNA. As a result of the spectrophotometry, samples above  $23 \text{ ng } \mu\text{l}^{-1}$  were diluted to  $4 \text{ ng } \mu\text{l}^{-1}$  prior to the inhibition assay. Evidence suggests that spectrophotometry is compromised when used with soil DNA as humic acids absorb light at wavelengths used to analyse DNA. Consequently, an inhibition assay was designed to detect, and quantify the presence of inhibitors.

To carry out the inhibition assay, an inhibition standard was constructed that was added to the samples and amplified using a PCR assay. The standard was designed as a unique piece of DNA that should not occur in nature. When it was visualised the expected results were that the intensity of the amplified band would increase with a decrease in the amount of inhibition. Any samples that did not amplify were diluted until the inhibition standard was clearly visible on the gel. The band's intensity was measured, using the GelDoc system, to determine the extent of the inhibition and to compare the extraction methods.

#### Construction of inhibition standard

The method was adapted from the one used by Edwards *et al.* (2001). An internal standard was produced from the onion alliinase gene and pGEM-T plasmid DNA (Promega) to create a unique plasmid. Onion DNA was amplified using ONI 670 forward and reverse primers (Appendix 2) using conditions described in the General Materials and

Methods (Chapter 2) for the ITS PCR assay except the anneal temperature was 55°C and visualization of the products occurred on a 1% agarose gel. The bands were cut out of the gel and left in 200 µl of TE buffer for 16 hours at 4°C. The PCR product was purified from the gel using a purification kit (Wizard PCR Prep kit; Promega, UK). It was ligated into a pGEM-T vector (Promega) and transformed in *Escherichia coli* JM109 according to the manufacture's instructions. White colonies were grown in LB broth (Merck KGaA) overnight. Ten millilitres of the broth was purified using Wizard Plus SV Miniprep kit (Promega) to obtain pure plasmid with insert. The primers designed to amplify the standard (ONI 670/F and SP6 + 3) were used as they each anneal on to different parts of the standard. ONI 670/F anneals to the insert and SP6 + 3 anneals to the plasmid. Therefore, the primers are specific to this unique insertion event. A 10<sup>5</sup> fold dilution of the purified plasmid was used in the inhibition assay.

## Inhibition assay

The conditions used for the amplification of the inhibition assay were as for the detection of the presence of DNA in Chapter 2 except that 5 µl of the inhibition standard and 5 µl of each soil DNA extraction sample were added to each well with 15 µl of the PCR reaction mix. The water in the PCR reaction mix was adjusted to allow for this so that the final volume in each well remained 25 µl. The positive control was 5 µl of standard and 5 µl of water, the negative was 10 µl of water. The primers used were ONI 670 reverse and SP6 + 3 (Appendix 2) and the anneal temperature was 50°C. The products were electrophoresed using 2% agarose gel (0.05% ethidium bromide) and visualised using the Gel Doc system.

### 3.3.4 Statistical Analysis

All the measurements of band intensity obtained from the Gel Doc system were calculated as a percentage of the band intensity for the positive of the gel that they were electrophoresed in. Values of relative band intensity were transformed ( $\log_{10} [X+1]$ ) to normalise the data before they were subjected to analysis of variance using Genstat version 7.1 (Lawes Agricultural Trust, UK). To determine which samples were significantly different from each other the Tukey test was used. Those methods with a difference between the transformed means that was greater than the T value ( $p = 0.05$ ) were significantly different from each other.

### 3.4 Results

Spectrophotometry showed that the purity ratio for the methods varied. The range of purity ratio values were 0.63 to 1.55 for method 1A, were 2.07 to 2.22 for method 1B, were 0.79 to 8.04 for method 1C, 1.97 to 2.04 for method 1D, 2.05 and 2.67 for method 1E, 2.03 to 2.16 for method 1F, 1.17 to 2.26 for methods 1I and 1J, and 1.93 to 2.16 for method 1K. The purity ratio for method 2 were 1.70 to 1.93 and for method 3 ranged from 1.93 to 2.00.

The samples diluted to  $4 \text{ ng } \mu\text{l}^{-1}$  prior to being used in the inhibition assay were from method 1J; 3, 6 and 10, method 1K; 4, 5, 9, 10, and all the samples from method 3.

When samples were subjected to the inhibition assay, without being diluted to  $10^{-1}$  dilution, all those from methods 1A, 1C, 1E, 1F, and 1I resulted in no visible bands. All samples from methods 1B, 1C, 1D and 3 (diluted to  $4 \text{ ng } \mu\text{l}^{-1}$ ) resulted in smears being produced in the gel with no single band that could be distinguish. Samples from 1J and 1K diluted to  $4 \text{ ng } \mu\text{l}^{-1}$  resulted in smears whereas those that were not diluted did not produce any bands (For examples, see Figures 3.1 and 3.2). After diluting the samples to  $10^{-1}$  and  $10^{-2}$  inhibition standard bands were either not visible, visible or smears were seen (Table 3.1). Some samples produced measurable bands at a dilution of  $10^{-2}$  but were diluted to  $10^{-3}$  as the bands were not clear and there was some smearing apparent, indicating further dilutions were needed. All  $10^{-3}$  dilutions of the samples that were tested using the inhibition assay resulted in bands that were clear without any smearing. Samples from methods 1I and 3 were not diluted to  $10^{-3}$  as  $10^{-2}$  dilutions demonstrated clear bands of the expected size.

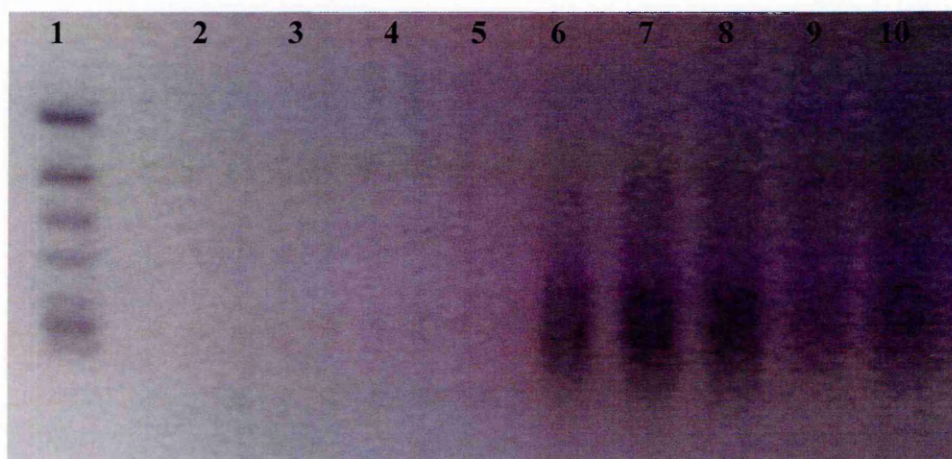


Figure 3.1: Demonstration of the inhibition assay with samples that resulted in either no bands or smears. Lane 1 contains ØX174 *Hinc*II cut ladder, lanes 2 to 5 are soil DNA samples that resulted in no bands and lanes 6 to 10 are soil DNA samples resulting in smears.

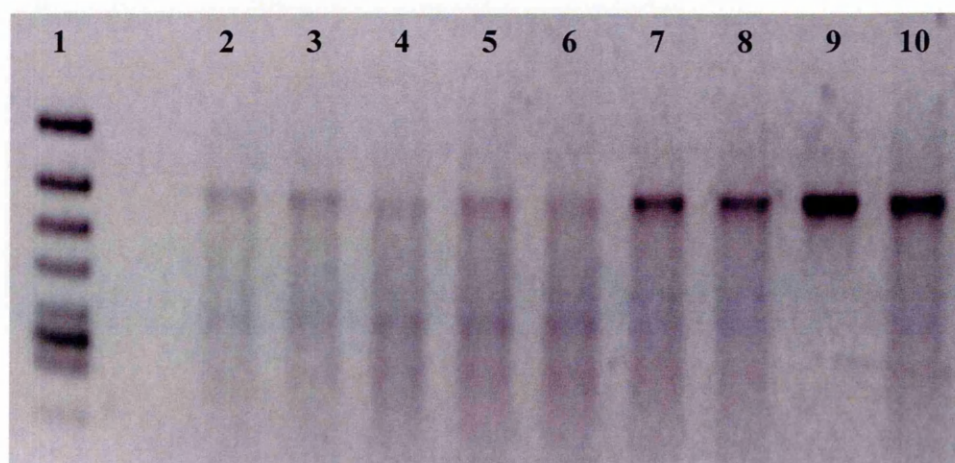


Figure 3.2: Demonstration of the inhibition assay with samples which resulted in measurable bands of variable strength. Lane 1 contains ØX174 *Hinc*II cut ladder and lanes 2 to 10 contain soil DNA samples resulting in bands of various strength.



Table 3.1: Presence (Y) or absence (N) of bands, or the presence of smeared bands (S), and the mean of the  $\log_{10}[X+1]$  of the relative band intensity (with the untransformed mean in parentheses) when samples diluted to  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$  were subjected to the inhibition assay.

Method	Dilution					
	$10^{-1}$		$10^{-2}$		$10^{-3}$	
	Bands	Measurable	Bands	Measurable	Bands	Measurable
1A	N	0(0)	S	1.626(68.6)	Y	1.886(77.3)
1B	S	1.578(55.2)	S	2.131(146.0)	Y	2.018(103.8)
1C	N	0(0)	Y	1.594(47.4)	Y	1.969(94.4)
1D	S	1.621(46.0)	Y	1.989(103.0)	Y	2.168(148.1)
1E	Y	1.060(11.2)	Y	1.615(41.1)	Y	2.101(126.8)
1F	S	1.170(18.0)	S	1.765(59.3)	Y	2.008(106.1)
1I	S	1.434(27.8)	Y	1.861(73.9)	Not diluted	Not Diluted
1J	Y	1.591(40.9)	Y	1.955(90.3)	Y	2.002(101.5)
1K	Y	1.551(36.9)	Y	1.974(95.3)	Y	2.031(107.1)
2	N	0(0)	S	1.710(51.3)	Y	2.038(109.9)
3	Y	1.942(92.7)	Y	1.926(84.6)	Not diluted	Not diluted

Analysis of the transformed ( $\log_{10}[X+1]$ ) values of the relative band intensity of the samples resulted in significant differences at  $F < 0.001$  between treatments at all three dilutions. Using the Tukey test ( $P = 0.05$ ) it was possible to establish which methods were significantly different from each other. The differences between extraction methods is shown in Table 3.2 for  $10^{-1}$  dilution, Table 3.3 for  $10^{-2}$  and Table 3.4 for  $10^{-3}$  (significant differences in bold). The T value for  $10^{-1}$  dilution was 0.2782, for  $10^{-2}$  it was 0.2819 and for  $10^{-3}$  it was 0.1298. All the tables appear to show that by adding an additional 0.2 ml of solubilization buffer the amount of inhibition is reduced, demonstrated by method 1A having a significantly lower transformed mean than 1B. Table 3.2 shows methods 1A, 1C and 2 have produced no bands suggesting that they are the methods that are least effective at removing the inhibition. Method 3, the CTAB method, being the most effective as it has a significantly higher mean from all the other methods. Methods 1E and 1F have

significantly lower means than 1D, 1J and 1K indicating that using a bead beater prior to the addition of solubilization buffer increased the efficiency of the method to remove inhibitors. In addition, method 1E is significantly different from 1I. 1F is not significantly different but the value in Table 3.2 is close to the T value. The theory that using a bead beater reduces the amount of inhibition is supported by the data in Table 3.3, where 1E is significantly lower than 1D, 1J and 1K, and 1F is significantly lower than 1J and 1K. Table 3.3 seems to show that method 1B is more effective at removing inhibition than methods that involve the addition of the solubilization buffer prior to bead beating. Table 3.4 indicates that method 1D, which involved adding 1.8 ml of the solubilization buffer, was the most effective at removing inhibition. In contrast, Table 3.3 suggests that method 1B was the most effective. Both methods 1B and 1D have a higher transformed mean, in Table 3.3, than method 3 but it is not significant. This might have indicated that 1B and 1D are more effective at removing inhibition than method 3 but Table 3.1 shows that at a  $10^{-1}$  dilution these samples produced smears where as method 3 did not.

Table 3.2: The differences between the means for dilution  $10^{-1}$  that were compared to the T value, 0.2782, derived from the Tukey test. Those differences greater than the T value are significant ( $p = 0.05$ ) and are shown in bold.

Methods	Methods									
	1B	1C	1D	1E	1F	1I	1J	1K	2	3
1A	<b>1.58</b>	0	<b>1.62</b>	<b>1.06</b>	<b>1.17</b>	<b>1.43</b>	<b>1.59</b>	<b>1.55</b>	0	<b>1.94</b>
1B		<b>1.58</b>	0.04	<b>0.52</b>	<b>0.41</b>	0.14	0.01	0.03	<b>1.58</b>	<b>0.36</b>
1C			<b>1.62</b>	<b>1.06</b>	<b>1.17</b>	<b>1.43</b>	<b>1.59</b>	<b>1.55</b>	0	<b>1.94</b>
1D				<b>0.56</b>	<b>0.45</b>	0.19	0.03	0.07	<b>1.62</b>	<b>0.32</b>
1E					0.11	<b>0.37</b>	<b>0.53</b>	<b>0.49</b>	<b>1.06</b>	<b>0.88</b>
1F						0.26	<b>0.42</b>	<b>0.38</b>	<b>1.17</b>	<b>0.77</b>
1I							0.16	0.12	<b>1.43</b>	<b>0.51</b>
1J								0.04	<b>1.59</b>	<b>0.35</b>
1K									<b>1.55</b>	<b>0.39</b>
2										<b>1.94</b>

Table 3.3: The differences between the means for dilution  $10^{-2}$  that were compared to the T value, 0.2819, derived from the Tukey test. Those differences greater than the T value are significant ( $p = 0.05$ ) and are shown in bold.

Methods	Methods									
	1B	1C	1D	1E	1F	1I	1J	1K	2	3
1A	<b>0.44</b>	0.10	<b>0.29</b>	0.08	0.07	0.17	0.26	0.28	0.01	0.23
1B		<b>0.54</b>	0.14	<b>0.52</b>	<b>0.37</b>	0.27	0.18	0.16	<b>0.42</b>	0.21
1C			<b>0.40</b>	0.02	0.17	0.27	<b>0.36</b>	<b>0.38</b>	0.12	<b>0.33</b>
1D				<b>0.37</b>	0.22	0.13	0.03	0.02	0.28	0.06
1E					0.15	0.25	<b>0.34</b>	<b>0.36</b>	0.10	<b>0.31</b>
1F						0.10	0.19	0.21	0.06	0.16
1I							0.09	0.11	0.15	0.22
1J								0.02	0.25	0.07
1K									0.26	0.03
2										0.05

Table 3.4: The differences between the means for dilution  $10^{-3}$  that were compared to the T value, 0.1298, derived from the Tukey test. Those differences greater than the T value are significant ( $p = 0.05$ ) and are shown in bold.

Methods	Methods							
	1B	1C	1D	1E	1F	1J	1K	2
1A	<b>0.13</b>	0.08	<b>0.28</b>	<b>0.22</b>	0.12	0.12	<b>0.15</b>	<b>0.15</b>
1B		0.05	<b>0.15</b>	0.08	0.01	0.02	0.01	0.02
1C			<b>0.20</b>	<b>0.13</b>	0.04	0.03	0.06	0.07
1D				0.07	<b>0.16</b>	<b>0.17</b>	<b>0.14</b>	<b>0.13</b>
1E					0.09	0.10	0.07	0.06
1F						0.01	0.02	0.03
1J							0.03	0.04
1K								0.01

### 3.5 Discussion

A numbers of samples extracted using methods 1J and 1K and all of the samples extracted using method 3 resulted in sufficient DNA that allowed the dilution of the samples to  $4 \text{ ng } \mu\text{l}^{-1}$ . This suggests that methods 1J and 1K can extract a larger quantity of DNA than other variations of method one. Method 3 would appear to always extract more DNA than the other extraction methods.

The spectrophotometry results suggest that the samples from methods 1A, 1B, 1D, 1E, 1F, 1K, and 3 do not contain pure DNA as the range of purity ratios does not include 1.8. Method 1C has a large range of ratios which demonstrates the variability between samples either of the humic acid which is affecting the ability of the spectrophotometer to measure the absorbance of the DNA. Or the large range could be due to an inconsistent quality of DNA resulting from the same extraction method. As methods 1I, 1J and 2 have a narrow purity ratio range that includes 1.8 it would suggest that a number of these samples contain pure or close to pure DNA. Taking account of how close the values are to 1.8 and how large the range of ratio values are it could be expected that methods 1D, 2 and 3 are the most likely to contain DNA that would amplify under the PCR conditions. The problem with samples that are extracted from soil is the presence of inhibitors that are known to affect the efficiency of PCR amplification (Tsai and Olson, 1992a; 1992b). It has been noted in this project that although DNA samples may result in purity ratios close to the pure ratio of 1.8 they may not amplify. Cullen and Hirsch (1998) believed that humic acids can effect the spectrophotometry results obtained, and hence the calculation of a purity ratio. They showed that humic acids absorb ultra violet light at 260 nm. Any absorbance value for the DNA samples would be affected by the presence humic acids making it difficult to determine whether the results obtained were from humic acids and/or

DNA. The purity ratios calculated are therefore unreliable as a method of determining the purity of DNA extracted from soil. Fluorescent spectrophotometry (using dyes or stains) does offer an alternative way of determining concentration of DNA but it is only believed to be effective when the humic acid content is below  $250 \mu\text{g ml}^{-1}$ . It would be difficult to determine the humic acid content of every soil sample in a large experiment. The indication is that fluorescent spectrophotometry is not influenced by other chemicals that may be present in a DNA sample such as proteins, that absorb ultra violet light, and is specific to double stranded DNA (Cullen and Hirsch, 1998).

As the purity ratios calculated from spectrophotometry values were considered unreliable it was necessary to design a method to test the purity of DNA extracts using PCR amplification of a standard. The inhibition assay was designed to allow the quantification of the effect of the inhibitors on the amplification of the soil DNA by measuring the band intensity of the unique standard. The relative band intensity of the standard when it was amplified in the presence of the extracted soil DNA would indicate the effectiveness of an extraction method. Those methods that were effective at removing inhibitors would have a high relative band intensity. As the inhibition standard was developed from a unique insertion event it is extremely unlikely to occur in nature. This is in contrast to other assays used by Burgmann *et al.* (2001), Miller (2001), and Miller *et al.* (1999) where the DNA used as a standard exists in nature although not in the environment where the sample DNA was taken from. The fact that this study used DNA that would not be found in nature means that this assay may be more reliable as it is not at risk from contamination from other environmental samples. A recent study (Klerks *et al.*, 2004) used an internal amplification control (IAC) in a real-time PCR to assess the efficiency of the PCR and determine the presence of false negatives. These authors used pure cultures of *Salmonella enterica* and *Escherichia coli* 0157:H7 which may not contain PCR inhibitors

whereas soil samples might. They believe that the sensitivity, precision and accuracy of the inhibition assay may be affected when environmental samples are tested.

In this experiment none of the methods tested produced bands which were distinguishable when the DNA extracts from soil were amplified without any dilutions indicating that the level of inhibition was such that it prevented the amplification of the inhibition standard. This would suggest that none of the methods were successful in removing all the inhibitory compounds in the soil. In fact methods 1A, 1F and 2 contained such a high content of inhibiting substances that no bands were produced. Dilution of samples has been shown to increase the likelihood of the DNA amplifying as the inhibiting chemicals are being diluted (Volossiuk *et al.*, 1995).

Method 3 appeared to be the most effective extraction method. This is supported by the work completed by Zhou *et al.* (1996) who believed CTAB to be effective at removing humic acids. Method 1C did not produce any bands at  $10^{-1}$ , indicating this was not a successful adjustment to the original method but it does have another practical reason for being used. In this method the samples are centrifuged, and the supernatant is aliquoted into a clean tube prior to the addition of the DNA binding resin. This removed particles within the soil organic matter that blocked the filter. Centrifugation of samples through the filter without this step increased the length of time required. This problem can further inhibit the process as increased lengths of spinning increase the risk of drying out the filter – which is not recommended by the author of the method. Presumably the action of drying the filter (as recommended after the addition of the ethanol wash) and subsequently centrifuging liquid through the filter facilitates the release of the DNA.

Method 2 resulted in no bands being produced at  $10^{-1}$  dilution. A number of studies have used this kit, and found it effective in extracting DNA. Herdina *et al.* (2004) used three extraction methods while attempting to quantify *Ggt* in soil by slot-blot hybridization. They tested two methods that involved the use of EDTA and SDS, as well as

this kit, to extract DNA from soil organic matter. Their conclusion was that it did not influence their results. The results in this chapter suggest that PCR is more sensitive to inhibitors present within soil organic matter than the DNA hybridization used by Herdina *et al.* (2004). The inhibition assay in this study suggested that the UltraClean<sup>TM</sup> Soil DNA isolation kit was one of the worst methods tested. This is in contrast to experiments where the UltraClean<sup>TM</sup> kit was compared with methods that included the use of CTAB, EDTA, SDS and bead beaters which indicated that it was an effective method at extracting DNA that would amplify in a PCR (Stach *et al.*, 2001). They reported that the DNA that had the highest purity was extracted by the kit. Although Braid *et al.* (2003) used the kit with a number of soil types and found that DNA from seven of the twenty soil types tested contained inhibition when amplification was attempted using 16S rRNA primers in a PCR. These studies extracted DNA from soil rather than soil organic matter, used by Herdina *et al.* (2004), and in this study.

Tsai and Olson (1992a) demonstrated the impact of various concentrations of humic acids, as little as  $0.1 \mu\text{l ml}^{-1}$  resulted in no PCR amplification. An attempt to establish what the humic acids were inhibiting was inconclusive. Humic acids are known to chelate copper, iron (Stevenson, 1994), magnesium (Tsai and Olson, 1992b) and other ions. It was thought that by binding with the magnesium in the PCR assay the Taq polymerase would be inhibited. Tsai and Olson (1992b) suggested that this could not be the case as no product was seen when a high concentration of the ion was used in the assay with a low concentration of humic acid. Tebbe and Vahjen (1993) found that by changing the polymerase that they used, they were able to amplify products from samples with higher concentrations of humic acid. When Smalla *et al.* (1993) changed the polymerase they were using from AmpliTaq (Perkin-Elmer/Cetus) to SuperTaq (Sphaero-Q) they obtained results from increasing amounts of soil DNA per reaction volume. Although they still obtained smearing, as in the experiment in this chapter, the cause of which they could not

determine, they found that by using a Stoffel fragment, and sometimes adding glass milk or spermine, distinct bands would be visualised. A suggested reason for this may be that these products chelate to the inhibitors, removing them from their association with the DNA. Consequently, the DNA is free for amplification. The smearing could possibly be some sort of combined product of the DNA and the inhibitors. Perhaps the presence of other soil components influences the interaction between humic acids, DNA and the polymerases. This would suggest a reason for different results obtained with different soil types (Braid *et al.*, 2003), beyond the variable amount of humic acids in soils. Many authors believe that a further purification step is necessary to remove this inhibition. Miller (2001) concluded that Sepharose columns were the best, Zhou *et al.* (1996) used a Wizard PCR Preps purification resin (Promega, Madison, Wis.) and Kuske *et al.* (1998) used a Sephadex G200 microcolumn. As the amount of extraction and purification increases so does the cost, making these options less viable for a commercial assay.

As previously mentioned none of the filter methods including the UltraClean kit were as effective as the CTAB method at reducing inhibition in the samples. This was the best method for this extraction although some dilution was still needed to reduce the effect of the inhibition present. Any dilution of samples not only dilutes the inhibitors but dilutes the DNA. As the DNA is diluted the chance of any desired product being amplified reduces, and consequently the sensitivity of the PCR is reduced. Inhibition, in the form of humic acids, has been seen to reduce amplification efficiency of PCR (Tsai and Olson, 1992a). Obtaining pure DNA that will amplify is difficult and purification is often thought to be necessary (Miller, 2001), making it prohibitive for a commercial assay. There are various different soil types, and samples can vary in their humic acid content. Studies have shown that DNA from different soil types may result in different amplification results, probably due to variable concentrations of inhibitors, when subjected to the same DNA extraction methods (Braid *et al.*, 2003). It could be suggested that extraction methods



require adapting for every soil type. This would mean that when surveying of soils from various locations that are of a different soil type would take more time. It would be unrealistic to have a commercial assay which meant that a number of different extraction techniques had to be used. In addition, soil is a bulky and heavy medium to collect, reducing the ease of sampling. All of these points make the use of soil unattractive for a commercial assay. A method which used plant material to detect the take-all pathogen's DNA would be easier to collect, handle and process. It is a method that has already been applied to a number of other diseases (for example, Nicholson *et al.*, 1996). Bateman *et al.* (2000) suggested that *Tapesia yallundae*, *T. acuformis*, *Rhizoctonia cerealis* and, *Microdochium nivale* vars. *nivale* and *majus* DNA followed a similar pattern to visual disease assessments over a growing season, responding to treatments in the same way. This may mean that it is possible to use DNA concentration, established by competitive PCR, to measure the extent of infection in a crop through the season. If the possible yield loss or disease severity could be predicted from the concentration of pathogen DNA in the previous crop then agronomic decisions could be adjusted to take this into account.

## **Chapter Four**

**Development and validation of a competitive  
PCR assay for *Gaeumannomyces graminis* var.  
*avenae* and var. *tritici*, the causative agents of  
take-all in wheat**

## 4.1 Introduction

PCR has been studied as a possible method of identifying the pathogen causing take-all in wheat plants. As early as 1991 (Schesser *et al.*) primers were designed to identify take-all in wheat seedlings. The primers KS1F, KS2R, KS4F, and KS5R were used in a nested PCR where KS1F and KS2R amplified *Gaeumannomyces* species DNA in an initial PCR. KS4F and KS5R were then used to amplify the product of the first PCR reaction. Although these primers appeared to be effective in amplifying DNA they appeared to be only genus-specific. As a result these authors used a probe, pMSU315 (Henson, 1989), to confirm that the product DNA was from *G. graminis*. Henson *et al.* (1993) used the PCR protocol developed by Schesser *et al.* (1991) but increased the annealing temperature of the first round of amplification and did not use the probe pMSU315. In addition they did not dilute the reaction products from the first round of amplification to reduce contamination. To reduce 'primer-dimers' they used 'hot-start' PCR where the samples are added to the thermal cycler after it has reached the first denaturing temperature. This study resulted in 188 bp fragments being produced from *G. graminis* mycelium DNA samples. Plant samples that were thought to be infested with take-all, when amplified with these primers also produced bands of 188 bp. Interestingly, when soil was added to the fungal samples the PCR assay was inhibited.

Ward (1995) demonstrated that by increasing the annealing temperature when using the primer pair KS1F and KS2R, it was possible to use them in a single round of amplification rather than in a nested PCR. The increase in annealing temperature was also found to reduce the occurrence of false positives. These primers were able to amplify all three varieties of *G. graminis*. Fragments for *Gga* and *Ggt* were both 600 bp long whereas the fragments amplified from the variety *Ggg* were approximately 50 bp smaller. This

means that a distinction between PCR products from *Gga* and *Ggt* varieties could not be made.

Ophel Keller *et al.* (1995) tested the ability of the primer pairs KS1F:KS2R and KS4F:KS5R, in a nested PCR, to amplify *G. graminis* in soil. They found that this PCR assay was able to demonstrate the presence of the fungus in samples that had a disease rating of greater than 21% of seminal roots infected. It was found that when the disease rating was as low as 7%, consistent results were not obtained. These primers were tested in a nested PCR with a number of other soil fungi and only three produced a band. *Phoma medicaginis*, *Penicillium* sp. and *Rhizopus* sp. produced a band of 188 bp after the first amplification. The *G. graminis* isolates produced two bands of 287 and 188 bp.

Ward and Bateman (1999) tested the primers KS1F and KS2R with other *Gaeumannomyces* and *Phialophora* species. They showed that these primers amplify *G. graminis* varieties, to produce bands of 600 bp with the varieties *Gga* and *Ggt* and bands of between 435 and 550 bp for *Ggg*. Other *Gaeumannomyces* and *Phialophora* isolates also produced bands of 550 bp.

Bryan *et al.* (1995) looked at the *Gaeumannomyces-Phialophora* complex and compared ribosomal sequences of various species, including the different varieties of *G. graminis*. They found that it was possible to distinguish between the different varieties using the internal transcribed spacer (ITS) sequences. Consequently, they were able to design variety-specific primers for a diagnostic PCR assay. They tested the primers pGt1 and pGt2 with 11 isolates of *Ggt*, a number of *Gga* isolates, wheat roots infected with both *Ggt* and *Gga* and healthy wheat roots. Amplification resulted in the expected 356 bp band with the *Ggt* isolates and wheat samples infected with *Ggt* and no bands with the others. When these primers were tested with other related fungi no band was produced.

Fouly and Wilkinson (2000) developed a reverse primer (GGT-RP) that could be used with the universal primer NS5 to detect the presence of *Ggt*. To develop the primer GGT-RP the primers NS5 and NS6 were used to amplify the 18S subunit of rDNA. The

amplified sequences were aligned to find a primer specific for the variety *tritici*. Using the primer pair of GGT-RP and NS5 it was found to be possible to amplify fragments from *Gga* and *Ggt* but not *Ggg* or other fungi tested. The primers were used for infected tissue as well as cultures. Amplification of *Ggt* isolates resulted in a fragment of 410 bp, *Gga* isolates resulted a 300 bp product. A reverse primer was also developed for the amplification of *Gga* isolates (GGA-RP) to produce a 400 bp product from *Ggt* and *Gga*. These authors suggest that the amplification of the 18S region of rDNA may be more consistent than the ITS region as the ITS region is highly variable between *Gaeumannomyces* species.

More recently, another set of primers have been developed to amplify *Ggt*, *Gga* and *Ggg* DNA. Rachdawong *et al.* (2002) developed three forward primers that could amplify avenacinase-like genes of each of the varieties of *G. graminis* with a single reverse primer. These forward primers were designed to be used together in a multiplex PCR assay. The amplification of each fungal variety using the primers resulted in PCR products of different sizes, the *Gga* primer gave a 617 bp product, the *Ggt* primer gave a 870 bp product and the *Ggg* primer resulted in a 1086 bp product. A number of other fungi were tested with these primers but no bands were produced. The fungal isolates used for this study were limited to the USA and as a result the primers may not work in the UK. This is due to the fact the *G. graminis* populations may differ across geographical regions. In addition, the primers amplify DNA from the avenacinase-like gene in contrast to other primers, which amplify within the ribosomal or mitochondrial DNA. It is likely that these primers would not be as sensitive as those that amplify large copy number DNA.

PCR can be used to identify the presence of a pathogen but it is often necessary to assess the amount of inoculum within a sample as this can be used to determine the amount of disease (Ophel Keller *et al.*, 1995). In 2000, Herdina *et al.* used a DNA slot-blot hybridization assay to quantify *Ggt* in soil. The authors compared the results of the assay

on soil with those of known concentrations of the fungus but as previously mentioned the correlation was poor.

It is possible to quantify the amount of DNA within a sample using competitive PCR (Edwards *et al.*, 2001). To quantify the amount of target DNA in a competitive PCR the amount of the target PCR product is compared to the PCR product of an internal standard added at a fixed concentration. The internal standard is added to each reaction tube and competes for the same primers as the target DNA. The target and internal standard PCR products are different sizes, so they can be distinguished by gel electrophoresis. As a result, both bands can be measured for their light intensity and the ratio of the two is compared to a standard curve. The standard curve consists of a known concentration of the internal standard amplified with a serial dilution of the target DNA. By including a known concentration of an internal standard into the reaction tube it is possible to remove the variability that occurs between each tube (Zimmermann and Mannhalter, 1996).

If it was possible to quantify the take-all pathogens of wheat, using a competitive PCR, then it may be possible to correlate pathogen DNA concentration with disease symptoms, or yield, so a disease prediction model could be designed. To do this primers are needed which are designed to amplify take-all DNA. Published primers would have to be tested for their suitability for use in a competitive PCR. If published primers are not suitable then primers would have to be designed from known DNA sequences found in databases and tested with known take-all isolates. The new primers could then be used to design an internal standard to amplify within a competitive PCR assay. To establish whether there is a relationship between the concentration of pathogen DNA and disease symptoms and yield, these would have to be measured in a crop of wheat. By measuring these data it would be hoped that a link could be found that would allow the prediction of disease symptoms or yield loss from the concentration of DNA earlier in the season. A correlation must be found before any prediction model is proposed as it is not known how

the concentration of DNA increases in relation to the symptoms and yield loss that is observed. Being able to predict the effect of take-all on a crop from the initial inoculum would allow agronomic decisions, such as the application of fungicides, to be made. The advantage of using a PCR based method is that it can be completed more quickly than conventional methods such as a bioassay. PCR assays are known to detect pathogen DNA prior to the development of symptoms, and multiple pathogens can be detected in a single PCR assay.

## **4.2 Aims**

- To identify PCR primers and conditions which can identify the take-all pathogens of wheat
- To design a competitive PCR assay to quantify the take-all pathogens of wheat based on the above
- To validate the competitive PCR assay using a number of field samples
- To establish if a relationship exists between the DNA concentration of take-all pathogens and take-all disease symptoms and/or yield loss.

## 4.3 Materials and Method

### 4.3.1 Evaluation of *G. graminis* primers

Take-all isolates were obtained from Dr G. Bateman at IACR – Rothamsted (Table 4.1).

Table 4.1: *G. graminis* isolates supplied by Dr G. Bateman

Isolate	Variety	Host	Location	Date	Reference
P086/441	<i>avenae</i>	wheat	Ireland	1986	Ward and Gray, 1992
P086/439	<i>avenae</i>	oats	Ireland	1986	Ward and Gray, 1992
91.A1.1	<i>avenae</i>	Not known	Not known	Not known	Not known
YZ2	<i>avenae</i>	Not known	Not known	Not known	Not known
91B2.3	<i>avenae</i>	Not known	Not known	Not known	Not known
89/5-1	<i>graminis</i>	wheat	Herts., UK	1989	Ward and Gray, 1992
89/5-3	<i>graminis</i>	wheat	Herts., UK	1989	Ward and Gray, 1992
93F2.1	<i>tritici</i>	Not known	Not known	Not known	Not known
0g12N	<i>tritici</i>	Not known	Herts., UK	1960	Ward and Gray, 1992
88/10-4	<i>tritici</i>	Not known	Not known	Not known	Not known
93E4.2	<i>tritici</i>	Not known	Not known	Not known	Not known

Isolate maintenance, fungal DNA extraction, spectrophotometry and ITS PCR assays were performed as detailed in Chapter Two (Page 32). The DNA was diluted to 1 ng  $\mu\text{l}^{-1}$ .

DNA from each isolate in Table 4.1 was used to establish whether published primers, in Table 4.2, designed for the amplification of *G. graminis* varieties would



amplify the isolates under standard PCR conditions employed at Harper Adams University College (Edwards *et al.*, 2001). The PCR assays for each primer pair were repeated once.

Conditions for the amplifications with the different primer pairs were the same as for the ITS 4 and ITS 5 amplifications except the annealing temperatures were adjusted according to the published methods (Table 4.2).

Table 4.2: Primer pairs tested under conditions at Harper Adams University College. Primer sequences are detailed in Appendix 2.

Forward Primers	Reverse primers	Anneal Temperature (°C)	Expected size of product (bp)	Position of primers	Reference
pGt1	pGt2	55	356	ITS 1 and ITS 2	Bryan <i>et al.</i> (1995)
KS1/F	KS2/R	60	600 ( <i>Gga</i> and <i>Ggt</i> )	Ribosomal DNA	Ward (1995)
NS5	GGA-RP	52	400	18S ribosomal DNA	Fouly and Wilkinson (2000)
NS5	GGT-RP	52	410	18S ribosomal DNA	Fouly and Wilkinson (2000)
<i>Gga</i>	AVE/R	58	617	Avenacinase-like gene	Rachdawong <i>et al.</i> (2002)
<i>Ggg</i>	AVE/R	58	1086	Avenacinase-like gene	Rachdawong <i>et al.</i> (2002)
<i>Ggt</i>	AVE/R	58	870	Avenacinase-like gene	Rachdawong <i>et al.</i> (2002)
HAGG/F	HAGG/R1	50, 52, 55	373	ITS 1 and ITS 2	Wilson <i>et al.</i> (this chapter)
HAGG/F	HAGG/R2	50, 52, 55	393	ITS 1 and ITS 2	Wilson <i>et al.</i> (this chapter)
HAGG/F	HAGG/R3	50, 52, 55	405	ITS 1 and ITS 2	Wilson <i>et al.</i> (this chapter)

Further testing of HAGG/F and R3 was completed by testing fungi isolated from infected roots (for methods see Chapter Two, page 33), which were identified as *G. graminis* or otherwise based on morphological characteristics. Additional *Ggt* and *Gga* isolates from G. Bateman at Rothamsted were tested with the HAGG primers. A range of

other wheat pathogens from the Harper Adams University College collection were also tested. A list of all isolates collected is provided in Appendix 1.

#### **4.3.2 Primer design**

To design the primers specific to *Ggt* and *Gga* a search was completed for *Gaeumannomyces* internal transcribed sequences, which had been deposited in the EMBL database (<http://srs.hgmp.mrc.ac.uk/>, 10/12/01). These sequences were aligned using CLUSTAL-W (<http://workbench.sdsc.edu/>, 10/12/01). Once aligned primers were designed from the sequences to bind to regions where there were differences between the varieties. Primer sequences were checked for homology to other sequences within GenBank using BLAST software (<http://workbench.sdsc.edu/>, 21/12/01). A web package, <http://www-genome.wi.mit.edu/cgi-bn/> (10/12/01), was used to assess the complementarity within and between the primers, the base pair content and the melting temperature.

#### **4.3.3 Development of the internal standard**

The method followed was the one used by Edwards *et al.* (2001) where an internal standard was produced from onion alliinase gene. Firstly a 526 bp fragment of the onion DNA was amplified. Linker primers, consisting of ten bases from the 3' end of the HAGG/F and HAGG/R3 primers added to the 5' end of 14 bases of the onion DNA primers were used to amplify the onion DNA fragment. Two different Touch Up amplification programs were used. The PCR reaction mix was the same as previously but the first program (TVLIN41) consisted of 5 mins at 94 °C followed by 10 cycles of 30 secs at 94 °C, 30 secs at 42 °C and 60 secs at 72 °C. A further 25 cycles with an increased anneal of 55 °C and an extension period of 5 mins at 72 °C was completed. The second program, LINK, involved an initial step of 90 secs at 94 °C. It was followed by 20 cycles of 30 secs

at 94 °C, 20 secs at 38 °C and 40 secs at 72 °C. After these cycles the samples were subjected to 10 cycles of 20 secs at 94 °C, 20 secs at 50 °C and 80 secs at 72 °C. The products from both amplifications were visualized after electrophoresis on a 2% agarose gel. The bands were cut out of the gel and left in 1 ml of TE buffer for 16 hours at 4 °C. The DNA in the TE buffer was amplified with the HAGG primers using the LINK program and the resulting products were cut out of the gel and left in 1 ml of TE buffer for 16 h at 4 °C. Five µl of the DNA in the TE buffer solution was amplified using standard conditions for the HAGG primers and the annealing temperature of 52 °C. After the amplification a gel slice containing the PCR band of 566 bp (the internal standard) was removed from the gel and the internal standard was purified using a Wizard PCR Prep kit according to the manufacture's instructions (Promega, UK). It was ligated into a pGEM-T vector and transformed into *Escherichia coli* JM109 according to the manufacturer's instructions (Promega). White colonies were tested for presence of the internal standard using the PCR protocol for the HAGG primers using a 52 °C annealing temperature. Positive colonies were grown in LB broth (Merck KGaA) overnight. Ten ml of the broth was purified using Wizard Plus SV Miniprep kit (Promega) to obtain pure internal standard plasmid DNA. A ten-fold dilution series of the internal standard and a two-fold dilution series (using a stock concentration of 1 ng µl<sup>-1</sup>) of the *Ggt* isolate 93F2.1 were made. These dilutions were amplified independently, and then together, under standard conditions for the HAGG/F and R3 primers to assess the intensity of the bands they produced. This allowed the determination of the concentration of internal standard which resulted in a standard curve which had the greatest sensitivity and broadest range. The internal standard plasmid preparation stock was diluted 4 x 10<sup>-5</sup> fold in 20 ml herring sperm DNA (20 ng µl<sup>-1</sup>). This diluted stock was aliquoted 1 ml into 1.5-ml Eppendorf tubes and stored at -20°C. This stock was diluted 50:50 with TE buffer just prior to use.

#### 4.3.4 Quantitative competitive PCR of samples

The quantitative PCR assay consisted of 10 µl of internal standard, 10 µl of sample and 30 µl of reaction mix. The PCR reaction was the same as the diagnostic PCR except that 1 unit of *Taq* polymerase was used. To create the standard curve, 10 µl of internal standard was added to 10 µl of a fungal two-fold dilution series. Amplified bands were visualized after electrophoresis on an agarose gel using the GelDoc1000 system. Measurements of the intensity of the bands were taken from unsaturated images to determine a ratio between *Ggt* and internal standard PCR products. This was used to create a standard curve of *Ggt* concentration against PCR product ratio. The PCR product ratio of samples and the equation of the standard curve were used to calculate the concentration of target DNA within samples. The concentration of the target DNA was adjusted to take into account the total amount of DNA within the sample. The total DNA concentration of the diluted samples was established by spectrophotometric analysis. Therefore, results were calculated as picograms of take-all pathogen DNA per nanogram of total DNA (pg ng<sup>-1</sup>).

#### 4.3.5 Sampling of plants

Thirteen fields (six locally and seven outside the county) were selected by discussion with farmers as to which fields were going into second wheat, and which fields and how many could be sampled in the time frame. Five areas (squares 10 m x 10 m) were chosen within each field in locations that could be returned to later in the season. The areas were marked by stakes in the field margins, and were found on subsequent visits by triangulation. Six fields within Shropshire were sampled while the field was in stubble of

the previous crop, and growth stages 30, 39, and 69 (Zadoks *et al.*, 1974). The seven other fields were sampled while in stubble and at growth stage 69 (Table 4.3).

Fifty plants were taken from each ten metre square in a 'W' pattern. The plants were carefully removed from the ground in a manner which resulted in as much of the roots in the top five centimetres of the soil remaining attached to the stem. Any excess soil was shaken from the plants and washed plants were visually assessed. The fifty plants' stem bases and roots were combined. A sub-sample was taken and subjected to DNA extraction, using the method described in the General Materials and Methods (Chapter 2, page 37) and the competitive PCR described in this chapter.

Table 4.3: Coding, location, and growth stages that the fields were sampled.

Field Code	Location	Growth stages sampled
DIT	Shropshire	Stubble, 30, 39,69
PAV	Shropshire	Stubble, 30, 39,69
PAVE	Shropshire	Stubble, 30, 39,69
PRE	Shropshire	Stubble, 30, 39,69
WAL	Shropshire	Stubble, 30, 39,69
WIN	Shropshire	Stubble, 30, 39,69
BED	Bedfordshire	Stubble, 69
HALL	Leicestershire	Stubble, 69
HALN	Leicestershire	Stubble, 69
HUNT1	Cambridgeshire	Stubble, 69
HUNT2	Cambridgeshire	Stubble, 69
RUT1	Lincolnshire	Stubble, 69
RUT2	Lincolnshire	Stubble, 69

Ears were collected from each square at GS 85 – 89 (late dough stages to just before ripening) to estimate the yield. Ears were cut from five 50 cm lengths along randomly selected rows within each square/location. The ears were dried in an oven (Unitherm) (80 °C, 48 h), the mass was determined prior and post drying. The post drying mass was checked to establish that a constant mass had been achieved.

#### 4.3.6 Statistical Analysis

The concentrations of DNA, and TAR values were transformed ( $\log_{10} [X+1]$ ).

Simple regression analysis with groups was performed in Genstat version 7.1 (Lawes Agricultural Trust, UK) for all combinations of disease indices (TAR) and DNA quantities. Accumulated analysis of variance (ANOVA) was used to establish the amount of variability accounted for by the explanatory variate and the explanatory variate plus a group (field or seed treatment).

## 4.4 Results

### 4.4.1 Evaluation of *G. graminis* primers

DNA extracted from all isolates of each *G. graminis* variety produced strong PCR products when amplified with ITS 4 and 5 at 58 °C indicating the presence of fungal DNA of a quality and quantity suitable for PCR.

Table 4.5 shows that the primers designed by Bryan *et al.* (1995), pGt1 and pGt2 did not just amplify *Ggt* they also amplified all five isolates of *Gga* and one of the two *Ggg* isolates. The amplification of *Ggg* by these primers would result in them amplifying isolates which do not cause take-all in wheat. This was contrary to the authors' results, under their conditions these primers were seen to amplify just *Ggt* isolates, producing one band of 356 bp. These tests showed that a strong band smaller than the last band on the DNA ladder, and a larger band which was barely visibly, were produced when the *Gga* and *Ggt* isolates were amplified. Amplification of multiple bands by primers would mean that in a competitive assay there would be competition between all the target bands plus the internal standard resulting in PCR bands which were difficult to measure or interpret. The KS1/F and KS2/R primers also appeared to be unsuitable for use in the PCR assay as they did not amplify the *Ggt* variety isolates. From Table 4.4 it would appear that the primers designed by Rachdawong *et al.* (2002) and those designed by Fouly and Wilkinson (2000) are also not appropriate as they do not amplify the *G. graminis* DNA as expected. The Rachdawong *et al.* (2002) *Gga* primer amplifies both *avenae* and *tritici* varieties in contrast to the results obtained by the authors, under their conditions. In addition the *Ggg* primers amplifies none of the isolates that were tested and the *Ggt* primers only amplified two isolates, one *Gga* and one *Ggt*. Under the PCR conditions described GGA-RP and GGT-RP primers amplified sub-species of both of the two take-all causing isolates although they did not amplify one *Ggt* isolate. As all the primers did not consistently amplify the isolates

they were designed to and some resulted in multiple bands under HAUC standard PCR conditions it would appear that they are unsuitable for use within diagnostic PCR assays.

Table 4.4: Amplification of *G. graminis* isolates by published primers (+ = presence of a band; - = absence of a band; +/- = inconsistent result).

Isolate	Variety	Primers		AVE/R			GGA,	GGT,
		pGt1, pGt2	KS1/F, KS2/R	<i>Gga</i>	<i>Ggg</i>	<i>Ggt</i>	NS5	NS5
P086/441	<i>avenae</i>	+	-	+	-	-	+	+
P086/439	<i>avenae</i>	+	-/+	+	-	+	-	-
91.A1.1	<i>avenae</i>	+	-	+	-	-	+	+
YZ2	<i>avenae</i>	+	-/+	+	-	-	+	+
91B2.3	<i>avenae</i>	+	-/+	+	-	-	+	+
89/5-1	<i>graminis</i>	-	+	+	-	-	-	-
89/5-3	<i>graminis</i>	+	+	+	-	-	-	-
93F2.1	<i>tritici</i>	+	-	-	-	-	+	+
0g12N	<i>tritici</i>	+	-	-	-	+	+	+
88/10-4	<i>tritici</i>	+	-	-	-	-	+	+
93E4.2	<i>tritici</i>	+	-	-	-	-	+	+

#### 4.4.2 Primer design

As the published primers did not produce the expected bands new primers were designed from the ITS region of *G. graminis* sequences. The primers were called HAGG/F (AACTCCAACCCCTGTGAACA), HAGG/R1 (CGAGACCGCCGATGTTC), HAGG/R2 (TACTGCGTTCAGGGTCCTG), and HAGG/R3 (TTTACCGCGAGTTACTGCGT). The sequences that the primers are designed to amplify can be seen in Figure 4.1 (HAGG/F) and Figure 4.2 (HAGG/R1, R2, R3). HAGG/F was designed to amplify *Ggt* and *Gga* but not *Ggg*. The reverse primers were designed to complement HAGG/F. The comparison of the three reverse primers with HAGG/F with an annealing temperature of 50 °C showed that HAGG/R1 did not consistently amplify any variety (results not shown). Both HAGG/R2 and HAGG/R3 amplified the desired varieties, *Gga* and *Ggt*. In the case of HAGG/R3 there was a small amount of amplification of the *graminis* variety but this was possibly a result of a sub-optimum annealing temperature which is often associated with mispriming. These bands did not appear when the annealing temperature was increased to



52 °C. When the temperature was increased to 55 °C no bands were observed on the agarose gel. As a result the conditions needed for the diagnostic PCR were determined as in the ITS protocol but the HAGG/F and HAGG/R3 primers were used with an annealing temperature of 52 °C.

When HAGG reverse primers were used to amplify fungal DNA with ITS 5, R1 appeared to show no amplification products where as R2 and R3 amplified all the isolates. Conversely when HAGG/F and ITS 4 were used they amplified *Gga* and *Ggt* isolates (Table 4.5) suggesting that the forward primer is specific for DNA for wheat take-all causing fungi. Consequently HAGG/F and HAGG/R3 were selected as the optimum HAGG primer pair.

All the fungi that were isolated in this experiment and were suspected to be take-all causing fungi were amplified by the HAGG primers. All fungi that were isolated and believed to be non-take-all causing fungi were not amplified. An additional group of isolates from G. Bateman that were either *Ggt* or *Gga* varieties produced the expected band of 405 bp and the eyespot and *Fusarium* isolates from the Harper Adams collection did not produce a band.

Table 4.5: Amplification of *G. graminis* isolates by HAGG reverse primers (HAGG/R1, R2 and R3) tested with the forward primer (HAGG/F) at an anneal temperature of 52 °C. Reverse HAGG primers were amplified with ITS 5 and HAGG/F was amplified with ITS 4 at an anneal temperature of 50°C. (+ = presence of a band, - = absence of a band)

Isolate	Variety	Primers							
		ITS 4	R1		R2		R3		HAGG/F
		ITS5	ITS 5	HAGG/F	ITS 5	HAGG/F	ITS 5	HAGG/F	ITS 4
P086/441	<i>avenae</i>	+	-	-	+	+	+	+	+
P086/439	<i>avenae</i>	+	-	+	+	+	+	+	+
91.A1.1	<i>avenae</i>	+	-	-	+	+	+	+	+
YZ2	<i>avenae</i>	+	-	-	+	+	+	+	+
91B2.3	<i>avenae</i>	+	-	+	+	+	+	+	+
89/5-1	<i>graminis</i>	+	-	-	+	-	+	-	-
89/5-3	<i>graminis</i>	+	-	-	+	-	+	-	-
93F2.1	<i>tritici</i>	+	-	-	+	+	+	+	+
0g12N	<i>tritici</i>	+	-	-	+	+	+	+	+
88/10-4	<i>tritici</i>	+	-	-	+	+	+	+	+
93E4.2	<i>tritici</i>	+	-	+	+	+	+	+	+





### 4.4.3 Quantitative PCR standard curve

From the testing of the internal standard dilution series with the fungal dilution series it was established that a dilution of  $4 \times 10^{-5}$  of the internal standard was the most appropriate to use. A standard curve was successfully constructed (Figure 4.3 and 4.4) using the  $\text{Log}_{10}$  of product ratio (of the internal standard and fungal dilution series) and  $\text{Log}_{10}$  of the known concentration of target fungal DNA. The range of the standard curve is  $0.031$  to  $1 \text{ ng } \mu\text{l}^{-1}$ .

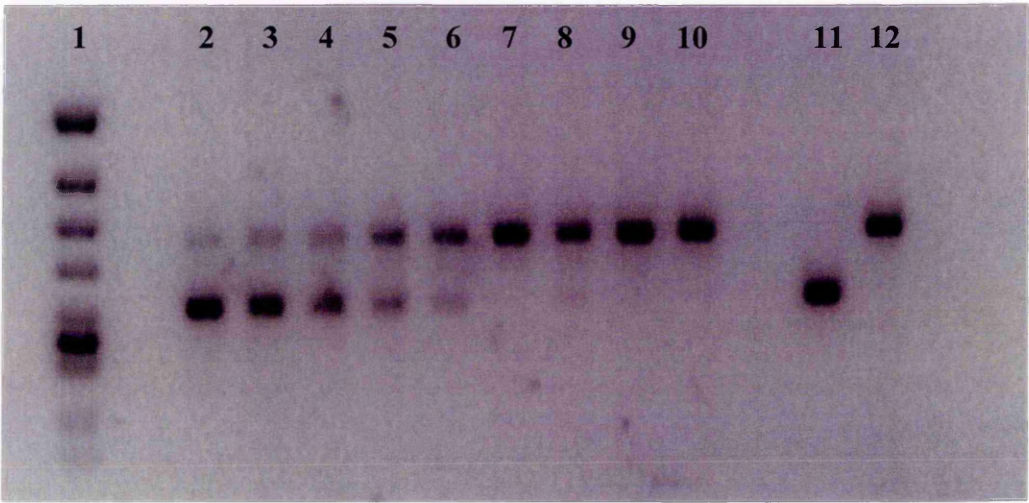


Figure 4.3: An example of the electrophoresis gel of the standard curve for *G. graminis* competitive PCR. Lane 1 contains  $\text{ØX174 Hinc II}$  ladder, lanes 2 to 10 contain two-fold dilution series of  $1 \text{ ng } \mu\text{l}^{-1}$  of *G. graminis* DNA with the internal standard, lane 11 contains *G.graminis* positive control and lane 12 contains negative control.

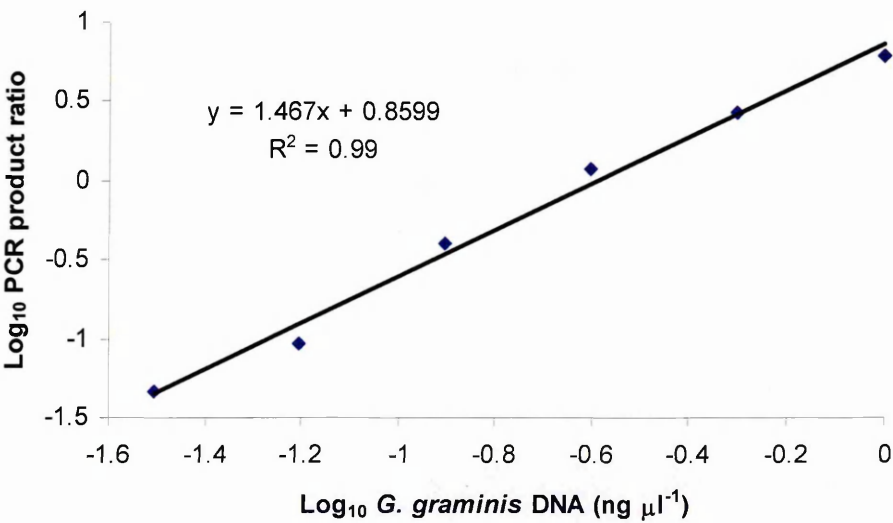


Figure 4.4: An example of a standard curve for *G. graminis* quantitative PCR

#### 4.4.4 Sampling of Plants

The competitive PCR assay (using the HAGG primers) amplified take-all pathogen DNA from samples that were extracted from plants collected from the commercial fields. It was possible to quantify this DNA using the HAGG standard curve.

When the stubble DNA was subjected to competitive PCR, plants from all fields, except HALN, appeared to contain some take-all DNA. It would appear that the take-all pathogen was present in all squares of the PAVE, WAL and HALL fields, but only present in some of the squares of the other fields. Pathogen DNA concentration from stubble ranged from 0.02 to 409.2 pg ng<sup>-1</sup>. The TAR values ranged from 0.5 to 40.5%. At growth stage 30 all the DNA that was subjected to PCR resulted in no amplification. All the TAR values for the stems were zero and the values for the roots were less than 3%. When the plants were assessed at growth stage 39 all the values for stem TAR were zero, two (6.7%) of the thirty values for the roots TAR were zero. The rest of the values were less than ten. Twenty-five of the thirty (83.3%) stem DNA samples were zero. Of the samples that did produce bands the concentration was calculated as less than 3 pg ng<sup>-1</sup>. Ten percent of the root samples resulted in no amplification. The field samples from DIT all had a concentration of less than 0.2 pg ng<sup>-1</sup>.

At GS 69 the samples for fields DIT and HALN all produced zero DNA values except HALN 3 which had a concentration of 0.27 pg ng<sup>-1</sup>.

Simple regression analysis was attempted to assess whether there was a relationship between the various data sets collected. As few correlations were found that had a significant percentage variance accounted for, simple regression with groups was used to establish whether the use of different fields in the sampling influenced the DNA concentration or TAR value. When comparing stubble DNA from the roots and stem the variability accounted for by the roots was 57% ( $p < 0.001$ , standard error of observation = 0.479) in an accumulated ANOVA. When the field was added to root DNA 71.9% of the

variability was accounted for, but field as a group was not significant ( $f = 0.051$ ). A larger variance accounted of 81% ( $p < 0.001$ , standard error of observation = 0.149) for TAR value of stubble roots was achieved when the TAR value for the stubble stem and roots was correlated (Figure 4.5). The accumulated variance for stubble roots plus field was 92% ( $p < 0.001$ ). The variance accounted for by the root DNA concentration at growth stage 69 when comparing the DNA concentrations for the stem and roots was 27% ( $p < 0.001$ , standard error of observation = 0.326). In this regression adding field as a group resulted in 57% ( $f = 0.005$ ) of the variability being accounted for.

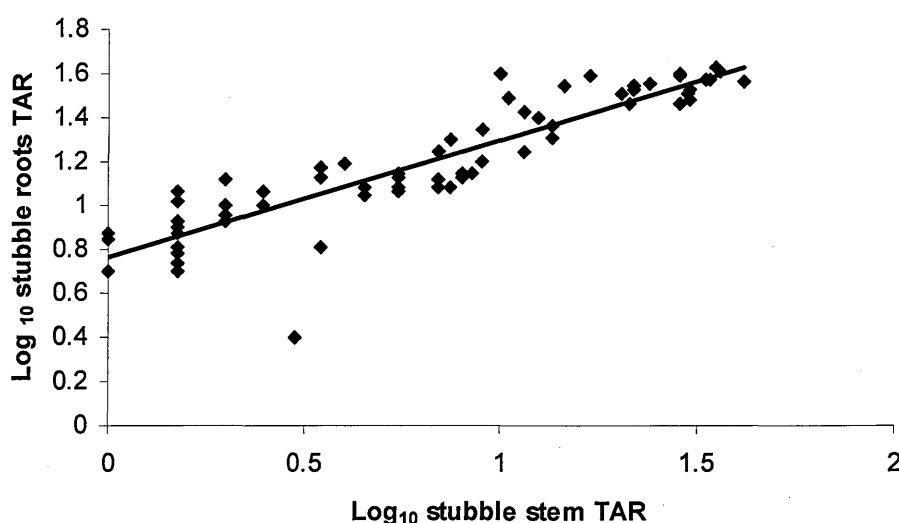


Figure 4.5: Regression of stubble TAR for stem and roots.

When correlating the TAR values for GS 69 roots against several other data sets a number of small but highly significant correlations were found. They were seen in regressions with take-all pathogen DNA concentration in stubble and at GS 69 samples, both roots and stems (Table 4.6). Accumulated ANOVA show that in these correlations when field was taken into account as a group, together with the explanatory variate, it accounted for an additional 43 to 54% of the variability. This is greater than the variability accounted for by the explanatory variates correlated with the TAR values for GS 69 roots.

Table 4.6: Regression analysis completed with the TAR values for GS 69 roots (response variate) and various explanatory variates.

Explanatory variate	Percentage variance accounted for	f value at P = 0.05	Variance accounted for when field was added	f value at P = 0.05	Standard error of observation
Stubble stem DNA	32.5	< 0.001	83.5	< 0.001	0.233
Stubble roots DNA	41.8	< 0.001	84.8	< 0.001	0.237
GS 69 roots DNA	32.6	< 0.001	86.6	< 0.001	0.477
GS 69 stem DNA	37.0	< 0.001	87.3	< 0.001	0.392

When correlating GS 69 root DNA with stubble take-all DNA and TAR for both roots and stems a number of highly significant correlations were observed (Table 4.7). In these regressions adding field in the accumulated ANOVA showed it accounted for an additional 40 to 63% of the variability.

Table 4.7: Regression analysis completed with the DNA concentration for GS 69 roots (response variate) and various explanatory variates.

Explanatory variate	Percentage variance accounted for	f value at P = 0.05	Variance accounted for when field was added	f value at P = 0.05	Standard error of observation
Stubble stem DNA	25.8	< 0.001	77.2	< 0.001	0.341
Stubble roots DNA	35.1	< 0.001	75.9	< 0.001	0.273
Stubble stem TAR	19.3	< 0.001	75.5	< 0.001	0.336
Stubble roots TAR	12.8	< 0.001	75.5	< 0.001	0.363

Further regressions with field as a group demonstrated it can account for an even greater amount of the variability. Table 4.8 shows it accounted for an additional 30 to 88% of the variability when added to the explanatory variate.

Table 4.8: Regressions with groups where 'plus field' has a greater affect than the explanatory variable.

Explanatory Variable	Response Variable	Variance accounted for by explanatory variable	f value at P = 0.05	Variance accounted for when field was added	f value at P = 0.05	Standard error of observation
Stubble stem DNA	Stubble stem TAR	2.9	< 0.001	91.2	< 0.001	0.177
Stubble stem DNA	GS 69 stem DNA	14.2	0.001	44.6	< 0.001	0.387
Stubble roots DNA	Stubble stem TAR	5.9	< 0.001	90.0	< 0.001	0.158
Stubble roots DNA	Stubble roots TAR	1.4	0.027	85.3	< 0.001	0.122
Stubble stem TAR	GS 69 stem DNA	2.2	0.157	46.6	0.002	0.366
Stubble stem TAR	GS 69 roots TAR	2.3	0.003	83.3	< 0.001	0.210
Stubble roots TAR	GS 69 stem DNA	1.1	0.312	44.7	0.002	0.365
Stubble roots TAR	GS 69 roots TAR	0.03	0.746	84.1	< 0.001	0.231

No regressions attempting to correlate the yield data with the various data sets for DNA concentrations and TAR values resulted in a percentage variance accounted for that was over 10%. A number of regressions demonstrated the explanatory variable plus field accounted for a greater proportion of the variability than any of the explanatory variables (Table 4.9).



Table 4.9: Regression analysis with groups completed with yield and various explanatory variates, where 'plus field' had a greater effect than the explanatory variate on yield.

Explanatory variate	Percentage variance accounted for	f value at P = 0.05	Variance accounted for when field was added	f value at P = 0.05	Standard error of observation
Stubble stem DNA	5.6	0.003	71.9	< 0.001	1.92
Stubble roots DNA	9.4	< 0.001	69.1	< 0.001	1.76
Stubble stem TAR	1.8	0.068	69.8	< 0.001	1.83
Stubble roots TAR	4.6	0.012	68.4	< 0.001	2.07
GS 69 stem DNA	0.7	0.188	69.4	< 0.001	1.61
GS 69 roots DNA	3.7	0.011	71.5	< 0.001	1.83
GS 69 roots TAR	3.8	0.012	71.0	< 0.001	1.89

## 4.5 Discussion

When developing the PCR assay it was necessary to find primers that would amplify isolates of *Ggt* and *Gga* that have been found to cause take-all of wheat in the UK. The primers should amplify all take-all isolates found in the UK; otherwise false negatives may occur. Equally it is important that the primers do not amplify varieties of *G. graminis* that do not cause take-all in wheat and that they do not amplify other fungi. If the primers amplified these organisms then false positives would result, which would mean that a take-all risk might be applied to a field that does not contain take-all pathogens. It is important that the primers amplify sequences that are conserved between *Ggt* and *Gga* because otherwise isolates will be amplified at a different efficiency. This would result in biased measurements of the quantity of DNA of the two take-all pathogens.

From this study it would appear that primers that have been published as amplifying varieties of *G. graminis* do not appear to amplify as the authors suggest using the standard PCR conditions used at HAUC. The most recently developed primers, AVE/R amplified with *Gga*, *Ggg* and *Ggt* (Rachdawong *et al.*, 2002), did not amplify as expected. The *Ggt* primer amplified only one of the *Ggt* isolates but also amplified one *Gga* isolate. This would mean that it would not necessarily identify the presence of all of the *Ggt* across the UK. To design these primers sequences of the avenacinase – like genes of isolates from the USA were used. As the UK and the USA are geographically separate may mean that there are genetic differences between the take-all causing fungi in the two countries. This has recently been demonstrated for an ear blight pathogen of wheat, *Fusarium graminearum* (O'Donnell *et al.*, 2000). These differences may be sufficient for primers designed on USA isolates not to amplify UK isolates. The sequences of the three UK isolates – *Ggt*, *Gga* and *Ggg* - used in the Rachdawong *et al.* study, including ATCC 28230, a *Ggt* isolate, differ from the other USA isolates by one base at the site where the *Ggt*-primer is designed to anneal. Consequently, this *Ggt* primer was not designed to

amplify ATCC 28230. Rachdawong *et al.* possibly thought that it was not necessary as they suggested that this isolate is more closely related to *Gga* than the other *Ggt* isolates used. Primers designed by Fouly and Wilkinson (2000) to detect *Ggt* and *Gga* also did not amplify as expected with cross reaction between the two varieties. As with the Rachdawong *et al.* primers, any variation in the PCR assay may influence the resulting products. The GGT-RP was designed on a region of the 18S ribosomal DNA that was not observed in the *Gga* isolates. Consequently, it would be expected that these primers would perform as detailed by the authors. With the primers not behaving as predicted they are not suitable for a diagnostic or quantitative PCR assay to be used in this study.

The pGt1 and pGt2 primers (Bryan *et al.*, 1995) amplified all the isolates tested except one *Ggg* isolate, 89/5-1. These primers are therefore inappropriate for use in the diagnostic or quantitative PCR assay because they would amplify isolates that do not cause take-all in wheat. The fact that multiple bands also occurred means that they are unsuitable for competitive PCR as this would affect the ability to quantify. The amplification efficiency of the target DNA and the internal standard DNA would be affected due to competition between them and the additional bands. When looking at how these primers have been designed it appears that they are based on a single base difference. This difference might not be enough to distinguish between the varieties, resulting in the amplification of all the varieties. When attempting to quantify the amount of *Ggt* and *Gga* a base difference between these varieties would probably result in the two isolates amplifying at different efficiencies. Although the results from this study suggest that a single base change is sufficient; the HAGG primers amplified these isolates strongly. Primer pairs based on one or two base pair differences between target and non-target DNA are highly dependant on optimum temperature and reaction conditions to achieve the required stringency. There is a chance that the genetic sequence of the isolates used in the study are different to those used by Bryan *et al.* resulting in the primers not amplifying the DNA of these isolates. To establish whether the isolates used in this study are genetically

different it would be necessary to sequence the regions of DNA that the Bryan *et al.* primers are designed to amplify and compare them for base differences. Research suggests that as there appears to be little gene flow between *Gaeumannomyces* populations any genetic change or difference between populations would be the result of genetic drift or selection pressure (Harvey *et al.* 2001). Consequently isolates from different locations may have evolved to have a different genetic sequence if different selection pressures have been applied.

Primers KS1/F and KS2/R (Ward, 1995) did not amplify as expected, as they were designed to amplify *Ggt* isolates and they amplified all varieties inconsistently. The original development (Schesser *et al.*, 1995) of these primers included testing only one isolate thought to have been from the USA but Ward tested 15 isolates which appear to be from the UK. One isolate, Og12N, was used by Ward and in this chapter. There is a contrast between Ward's results and those of this study, as Ward showed a 600 bp band being produced whereas in this chapter the primers failed to amplify Og12N. Again these conflicting results may be a result of differences in the PCR conditions employed.

As the published primers did not appear to amplify the isolates we used, new primers were designed and named HAGG/F, HAGG/R1 HAGG/R2 and HAGG/R3. When tested with the different isolates of *G. graminis*, HAGG/F with either HAGG/R2 and HAGG/R3 amplified *Ggt* and *Gga*, as expected. As a result both could be used for the diagnostic PCR. HAGG/R3 was chosen for the quantitative PCR because it amplifies the target DNA to produce the strongest bands with the *Ggt* isolates, and the bases that the primer was designed on were more conserved between sequences. Further analysis of the amplification of isolates using HAGG primers with ITS primers (Table 4.6) suggests that HAGG/F controls the specificity to *Ggt* and *Gga* demonstrated by the amplification of the *Ggt* and *Gga* isolates when HAGG/F and ITS 4 were used. As a result of the two varieties amplifying at a similar efficiency any infection of take-all in wheat should be detected with the primer set of HAGG/F and HAGG/R3. When this primer set is used to amplify *Gga*

and *Ggt* no visible difference can be seen in the band intensity. It was assumed that they amplify both with equal efficiency.

In 1992, a new variety of *Gg*, *maydis*, (Yao *et al.*) was identified on maize in China. Ward and Bateman (1999) attempted to determine the phylogenetic relationship between various *Gaeumannomyces* – like and *Phialophora* – like fungi. They sequenced DNA products from a PCR assay using ITS 4 and ITS 5. The resulting products contained DNA from the ITS regions and a number of bases from the 18S and 28S genes. Comparison of the sequences led the authors to believe that *Ggm* was so similar to *Phialophora zelicola* that they were the same fungi. The phylogenetic tree obtained by NEIGHBOR software suggested that these isolates were closely related to *Ggg* which would mean that they were unlikely to be amplified by HAGG primers.

The diagnostic PCR was successful in amplifying DNA from the fungal isolates from naturally infested plant material demonstrating the presence of take-all. The competitive PCR standard curve, with an  $R^2$  of 0.99 (Figure 4.3), demonstrated the ability of this assay to produce a standard curve in which there was a good relationship between target DNA concentration and the PCR product ratio. The extent of disease can not be assumed from the amount of DNA amplified as there is no reference as to what concentration of DNA is present when a certain level of symptoms is observed. To establish what concentration of DNA is observed when a particular level of symptoms is seen plant samples were collected from various fields in an attempt to determine the relationship. By comparing these data it was hoped that the assay could be used to establish what amounts of take-all DNA relate to what levels of disease. Results showed the competitive PCR was able to quantify take-all DNA from plant stem bases and roots collected from the field, when it was stubble, and at GS 39 and 69.

A number of correlations were attempted to identify the relationship between DNA concentration and symptoms (TAR). Few had high percentage variance accounted for, although most of the relationships were statistically significant so regression with groups

was used to establish whether factors connected with sampling different fields had a greater effect on the response variate than the explanatory variate did. Two regressions, stubble DNA roots with stubble DNA stems and stubble TAR roots with stubble TAR stems resulted in the explanatory variate accounting for over 50% of the variability seen in the response variate. This demonstrates that as the amount of disease increases in the roots so does the amount of disease in the stems, when measured by visual methods and competitive PCR. It is logical for a take-all to affect the roots first, disease on the stem developing later. Figure 4.4 appears to show this as for each point on the stem axis the roots axis value is higher. The accumulated ANOVA of these two regressions showed that when the field is taken into account the difference between the percentages is less than the percentage for the explanatory variate. This shows that in the case of these two regressions any effects of using different fields are less than the effect of the explanatory variate, stubble DNA or TAR for the roots.

Some of the other regressions attempted did show the explanatory variate having an effect on the response variate of over 12% but in all cases the increase in the percentage variance accounted for by the explanatory variate with the addition of field in the accumulated ANOVA was greater than that accounted for by the explanatory variate alone. In fact in many cases the explanatory variate was not significant but the explanatory variate plus field was. This indicates that field effects frequently have a greater effect on the disease or yield later in the season than any measure of inoculum. All of the regressions demonstrate that as the concentration of DNA increases so does the disease severity, but the relationships are weak making any ability to predict take-all disease based on earlier observations of DNA content or disease symptoms highly inaccurate. It was hoped that the yield may have a relationship to the concentration of DNA but unfortunately this was not the case. This was in contrast to work completed by Bateman *et al.* (2004) who demonstrated that take-all reduced yields. These experiments compared various seed treatments designed to reduce take-all yield loss in a crop. The result being that controlling

take-all reduced the loss. This contrast in results may be due to the effect of using different fields (in this study), as field effects often appear to be greater than any earlier disease observations on disease or yield later in the season. Bateman *et al.* (2004) used multiply sites but analysed them independently, so avoiding any inter-field differences.

Possible reasons for the lack of regressions is that the sampling technique did not allow an adequate representation of the take-all in an area. Sampling by its very nature does not result in the whole of the field being tested. The size of the sample can affect the accuracy of the data collected as the larger the sampling the greater the proportion of the whole field that is assessed. Sampling therefore has an impact on conclusions that are drawn from samples taken from a field (reviewed by Hughes, 1999). Diseases tend not to be distributed evenly across a field, take-all in particular is known for its patchy distribution, but taking large samples can inhibit an experiment or make it impossible to complete. It is necessary to balance the sampling protocols against the time available and the size of sample which can be processed. A 'W' walk within a field has been frequently used to detect the presence of disease and felt to be appropriate for this experiment (Delp *et al.*, 1986). Unfortunately, although disease was detected it did not appear to show a strong relationship to the concentration of DNA.

A number of other factors may have influenced the extent of the disease measured in this experiment including soil type and condition, and weather. Werker and Gilligan (1990) suggest that the variability they observed in disease severity in the three years of their experiments was due to different weather patterns in those years. With a 30% reduction in rainfall in April linked to lower disease and the disease developing quickly on roots but the number of infected plants decreased when a wet autumn was recorded. The authors suggest that this maybe a result of two opposing factors; enhanced establishment of disease and increased decay of the fungus. Any further spread of disease across a field would then be dependant on whether secondary infections were established and how the initial inoculum was spread in the field. These two opposing factors occurring at the same

time add to the complexity of this disease. These authors observed another factor which influenced the disease pattern – competition. They believed overcrowding caused increased vigor in a number of plants so the plants became more susceptible to infection.

The soil type is known to affect the severity of take-all. It is believed that heavy soils increase the likelihood of take-all reducing yield, whereas wheat planted on light soils is less likely to result in a severe epidemic, except where there is a high amount of organic matter. High organic matter is of concern as take-all is known to survive on plant debris. It spreads from plant to plant via mycelium. Certain weather conditions, such as warm wet periods (Cotterill and Sivasithamparam, 1987b), have been linked to an increase in degradation of the plant debris that the fungus lives, on reducing its ability to survive. The authors observed a decline in infective propagules with an increase in moisture content of the soil and temperature. Christensen *et al.* (1987) suggested that increased soil pH (from pH. 5.5 to 6.0) and inorganic soil nitrogen ( $\text{NO}_3^-$ ) would increase the prevalence of the disease. The variability of soil and weather conditions can affect the amount of initial inoculum and how that inoculum survives and spreads through a growing season but it does not explain the apparent lack of correlation between DNA concentration and visual disease symptoms observed in this study.

Another possible reason for the lack of correlation between the DNA concentration and the visual symptoms may be due to a problem with the PCR assay. Poor specificity of the primers may be a cause, resulting in the amplification of non- take-all fungi but this is unlikely as extensive searches of DNA databases were completed to establish whether they were likely to cross react. The result was negative as was the testing of the non-take-all isolates with the HAGG primers. Equally the DNA alignments and the testing of the HAGG primers with a large number of UK isolates suggests they are appropriately designed to amplify all take-all fungi in a wheat crop. Consequently, factors associated with host susceptibility and pathogenicity of the fungus is thought to be probably associated with the lack of a relationship between the data sets. A more susceptible variety



of wheat could be expected to develop symptoms more quickly and to a greater extent than a variety that is more resistant. Different cultivars of wheat have been seen to be colonized by *Ggt* at various rates supporting a theory that some varieties are susceptible to infection by the take-all fungus (Penrose, 1985). Golldack *et al.* (2004) observed that when *Ggt* was attempting to infect wheat plants, there was an increase in root growth and blocking of lesions. Further observations show an increase in number of roots with the primary infection which slows with time. Extensive primary infection may not result in high visual symptoms and a high loss in yield later in the year if the secondary infection is prevented or slowed by external factors such as a dry summer. Conversely the increase in root mass can lead to an increase in secondary infection as there is a large amount of inoculum available (Bailey and Gilligan, 2004). A host resistance mechanism of producing lignin has been seen when wheat roots were attacked by *Ggt* (Liu *et al.*, 2000), which may influence the progress of the fungus through the plant. This may explain the variability between the fields but still does not explain the lack of a consistent relationship between the DNA concentration and TAR values within field.

Variability between fields may also be attributed to the observation that the different fungicide treatments. Both Latitude (Knight, 2002) and Jockey (Dawson and Bateman, 2001) treatments have been found to control take-all, reducing the visual symptoms observed when compared to standard seed treatments such as Beret Gold.

A browning of the root tissue has been observed when no pathogen DNA was detected within them (Golldack *et al.*, 2004). These lesions have been proposed as a method of host resistance (Penrose and Neate, 1994). It is possible that brown lesions can be mistaken for those caused by disease in the visual assessment. This would affect the TAR value that was obtained. Although this author found that it was possible to distinguish between black take-all like lesions and brown lesions caused by other pathogens or the host defenses. The fact that lesions appear to be produced by host as well as by the pathogen

means that the need for PCR is highlighted to reduce confusion between what is host and what is caused by the pathogen.

There may be differences between the pathogenicity of the varieties *Ggt* and *Gga* to wheat, and differences in pathogenicity between isolates within the varieties. A take-all isolate that has the ability to overcome the host defences more quickly than another isolate would, in theory, be able to colonize a plant in less time. An increase in the activity of polygalacturonase has been seen in *Ggt* to correlate ( $r = 0.548$ ) with pathogenicity to wheat, measured as a reduction in shoot dry weight (Martyniuk, 1988). Those isolates that produced a greater amount of the enzyme could be expected to colonize plant more quickly resulting in more extensive symptoms and a reduction in yield. A variety of isolates in a field or between fields would make it difficult to correlate DNA to visual symptoms or TAR as some plants would develop symptoms quickly where as others may not, only have a small amount of symptoms. Another enzyme manganese lipoxygenase, produced by *Ggt* and *Gga*, has been linked to the breakdown of cell walls (Hornsten *et al.*, 2002). A laccase gene (LAC3) has also been observed to be transcribed in the presence of the host by *Ggt* (Litvintseva and Henson, 2002a; 2002b). It is possible that an isolate that produces a large amount of an enzyme than another isolate may be a more successful pathogen causing more severe symptoms (resulting in a greater TAR) and a large loss in yield.

At the completion of this experiment this author is unaware of any competitive PCR assay developed to quantify take-all DNA in plants material obtained from naturally infested fields. The experiments have been successful in this goal as it is possible to quantify take-all from samples of plants in stubble or at GS 39 and 69, taken from the fields selected. Establishing a relationship between the concentration of DNA and symptoms, as a TAR percentage, has been less successful. In fact the only strong relationship observed was that as symptoms increased in the roots of stubble, they increase in the stems. One year of experiments is not enough to show any consistent trends, so more experiments would need to be completed. To attempt to solve the problem of a lack of a

substantial relationship it would be prudent to attempt to find a way to assess host resistance and pathogenicity of isolates, as this may be a reason for the lack of correlation. If it was possible to take these factors into account in a measured way then a relationship may be observed. Soil and weather conditions also influence the progress of the disease through the season and may determine the extent of take-all within a crop. When a crop is drilled the weather conditions in the future season are not known. Although the soil type may be known, other soil conditions such as temperature and moisture content are not. These factors are known to influence the inoculum and hence the progress of the disease. As inoculum measured by competitive PCR assay prior to drilling did not correlate strongly or consistently with visual symptoms or yield later in the season its use in a decision support system is limited and not economically viable.

It may be possible to use the assay in field trials. A characteristic of take-all is its patchy distribution (Gams and Domsch, 1969) which makes siting field trials difficult. When testing the efficacy of fungicides it is beneficial to have a consistent disease pressure to enable comparisons to be made. Using the competitive PCR assay to determine pathogen DNA concentration could allow inoculum concentration to be taken into account in the analysis of take-all disease and yield during fungicides field trials.

## **Chapter Five**

### **Seed Treatment Field Experiment**

## 5.1 Introduction

In recent years a number of products have become available for the treatment of take-all in wheat. The treatments, Jockey (fluquinconazole and prochloraz), now also marketed as Gelmano, and Latitude (silthiofam), are applied as seed dressings and therefore, act as prevention rather than a cure. These chemicals are marketed as products specific for the prevention of take-all but Baytan (fuberidazole and triadimenol); an existing treatment is known to provide some protection from the disease.

Fluquinconazole applied as a seed treatment was shown to be effective in controlling take-all and other seed-borne and foliar diseases resulting in a 26% increase in yield (Wenz *et al.*, 1998). Initial investigation into this triazole fungicide indicated its activity against various Ascomycetes, Deuteromycetes and Basidiomycetes. Evidence suggested its activity against foliar diseases on apple but later indicated its possible use against foliar pathogens of wheat (Russell *et al.*, 1992). Yield increases of between 9 and 20% have been recorded (Lochel *et al.*, 1998) when fluquinconazole was applied to wheat seed. This chemical has been marketed as Jockey - a mix with prochloraz. Jockey has demonstrated the ability to increase yields in wheat when compared to Sibutol (bitertanol and fuberidazole) and Baytan (fuberidazole and triadimenol) (Bardsley *et al.*, 2000). This may be due to its activity against a number of different diseases as well as take-all. Studies have shown that Jockey was only cost effective to a farmer if it is used to treat a second or third wheat; where there was the greatest yield gain. Take-all in a first year of wheat appeared to be controlled by crop rotation. When an untreated crop followed a Jockey treated crop reduced yields were observed due to an increase in the amount of inoculum. The development of take-all decline was delayed by the application of the product (Bateman *et al.*, 2003a).

The treatment of seed appears to be most effective where the epidemic is severe with both Latitude (Schoeny *et al.*, 2001) and fluquinconazole (Bateman *et al.*, 2004). Latitude was shown to have activity against *Ggt* on medium and in growth room studies. Its activity was greater than the triazole, triadimenol (an active ingredient of Baytan). The specificity of this chemical to *Ggt* means that it has little or no activity against other pathogens and, as such seed is usually also treated with another seed treatment. In field experiments it demonstrated a significant reduction in take-all symptoms and whiteheads, with an increase in yield (Beale *et al.*, 1998a, Beale *et al.*, 1998b). Latitude's mode of action has been determined as the inhibition of the transportation of adenosine triphosphate from the mitochondria to the cytosol. The lack of energy stops any further growth (Joseph-Horne *et al.*, 2000). When Latitude, combined with fludioxonil, was compared to a mix containing fuberidazole and triadimenol (Baytan), and fludioxonil (Beret Gold) it appeared to give the best control of take-all (50% reduction in take-all severity in April) with the highest yield. On average, yield with Latitude treatment was  $0.46 \text{ t ha}^{-1}$  greater than the other treatments, when the crops were sown in September, and  $0.2 \text{ t ha}^{-1}$  when they were sown in October (Knight, 2002). In these experiments Baytan offered some protection from take-all by reducing take-all severity by 33% and increasing yield by  $0.36 \text{ t ha}^{-1}$  when the crop was sown in September. When the crop was sown in October Baytan did not affect the yield or disease severity. These authors believed that this product could be used where the disease risk is lower. Earlier work on triadimenol, a sterol biosynthesis-inhibiting compound, indicated its possible use in protecting plants from take-all. When a liquid form of the fungicide was mixed evenly with naturally infested soil in small pot experiments it inhibited the growth of the fungus demonstrated by at least a 88% reduction of disease on seedlings (Bateman *et al.*, 1990). A 3 to 11% reduction in disease was seen within plant populations when Baytan was applied to seeds drilled in naturally infested plots (Werker and Gilligan, 1990). These authors suggest that this reduction in disease may

be due to disease escape rather than fungicidal activity as Baytan was seen to reduce the number of roots by up to 6%, and hence the amount of disease. They observed that seed treatment did not have the greatest effect on disease – sowing date was the most significant factor in reducing disease symptoms. Although there was a reduction in visual symptoms, the effect of sowing date lessened through the year and in a number of years it disappeared all together.

Work completed by Herdina and Roget (2000) highlighted the importance of sampling. Fifty-six soil cores were taken from a nine hectare grid resulting in 28 kg of soil. They suggest it was necessary to take a sub-sample of 500 g from this 28 kg for every hectare sampled, for determining the risk of take-all (with a 95% accuracy). It was felt that by increasing the amount of soil sampled the accuracy of any conclusion drawn would increase. This is important when carrying out a large field experiment as it is necessary for the sampling to reflect the inoculum present and the population of plants in the experiment. Appropriate sampling can give a more accurate and therefore a more reliable picture of the epidemic within a field or plot. This is particularly relevant with take-all as it occurs in patches across fields. The heterogeneous nature of the disease can make representative sampling difficult. It is necessary to design experiments and sampling techniques to enable an accurate picture of the epidemic to be achieved. A 'W' sampling pattern for plants is believed to take account of variability that exists in a field (Delp *et al.*, 1986). Long, thin plots may also have the effect of reducing the variability between plots as they may be large enough to include both areas of diseased and non-diseased plants. A large scale field experiment was designed to test the efficacy of the seed treatments Baytan, Jockey and Latitude in controlling take-all in winter wheat. Long thin plots were used in an attempt to take account of the variability that occurs across a field.

## 5.2 Aims

- Determine the efficacy of Jockey, Latitude and Baytan seed treatments to control take-all in winter wheat in a large scale field experiment.
- Use the newly developed competitive PCR assay to quantify the take-all pathogen in wheat stems and roots throughout the season and compare it to visual disease assessments.



## 5.3 Materials and method

### 5.3.1 Establishment of the field experiment

Sixteen strips of 12 m by a maximum of 429 m (plots were of variable length due to shape of field) were marked out in a field near Much Wenlock, Shropshire (Landranger grid reference: SO 629985). The crops planted in this field prior to wheat were sugar beet in season 2001/2002 and winter wheat in season 2002/2003. The field was ploughed and power harrow immediately before drilling (3 m Accord pneumatic drill) on 9<sup>th</sup> October 2003 to a randomised block design with four replicates of four treatments at a seed rate of 168 kg ha<sup>-1</sup>. The seed treated was from one batch of Consort. The seed treatments were bitertanol (375 g l<sup>-1</sup> a.i.) and fuberidazole (23 g l<sup>-1</sup> a.i.) applied as Sibutol (Bayer CropScience Ltd., Cambridge, UK) at 1.5 l per tonne of seed, silthiofam (125 g l<sup>-1</sup> a.i.) applied as Latitude (Monsanto Ltd., Cambridge, UK) at 2 l per tonne of seed, fluquinconazole (167 g l<sup>-1</sup> a.i.) and prochloraz (31.2 g l<sup>-1</sup> a.i.) applied as Jockey (Bayer CropScience Ltd.) at 4.5 l per tonne of seed and, triadimenol (22.5 g l<sup>-1</sup> a.i.) and fuberidazole (22.5 g l<sup>-1</sup> a.i.) applied as Baytan (Bayer Crop Science Ltd.) at 2 l per tonne of seed. Sibutol was also applied to the Latitude treated seed at the same rates as above. Three metres at either edge of the strips was considered as discard, so that the plot was six metres wide, and ran the length of the experiment area (up to 429 m). All other agronomic inputs were applied according to standard farm practice and are recorded in Appendix 4.

### **5.3.2 Collection and assessment of plant material**

Two hundred and fifty stubble samples were collected on 3/10/03 from each plot prior to ploughing and 50 plants at growth stages 30 (2/04/04), 39 (28/05/04) and 69 (28/06/04), so that as much of the top five centimetres of the roots remained attached to the stem base. To collect the plant material a zigzag (a number of 'W' connected together) was walked along the each plot avoiding the three metres discards either side of plots. The plants were pulled out of the ground as evenly as possible along the length of the plot. Visual assessments, as described in Chapter Two (Page 35) and, molecular assessments were carried out on the plant roots and stem bases at stubble and, growth stages 30, 39 and 69. To establish the amount of take-all pathogen in the plant material the competitive assay developed in Chapter Four (Page 78) was used.

### **5.3.3 Harvest**

The experiment was harvested using a Lexion 480 (Claas) combine on 31<sup>st</sup> August 2004, by harvesting each plot. The yield was calculated for each plot by taking the mass of the grain on a weighbridge. Grain samples of 1 kg were taken from each plot. The grain samples were used to calculate moisture content, specific weight and thousand grain weight. Moisture content was established using a Grainmaster i (Protimeter, Shannon, Ireland) moisture meter. Specific weight was measured using an Easi-Grain Chonodrometer according to the manufacturer's instructions. Yield and 1000 grain weight were adjusted to 15% moisture content.

### 5.3.4 Statistical Analysis

A generalised analysis of variance (using Genstat version 7.1 (Lawes Agricultural Trust, UK)) was carried out on the raw values for yield, specific weight, 1000 grain weight and the  $\log_{10} [X+1]$  of all visual disease assessments and concentration of DNA. The transformations were completed to normalise the data. An analysis of variance for the same data was also completed with the untransformed values for the stubble TAR for the roots and stems and stubble DNA concentrations for the stems and roots, as a covariate. A significance level of 5% was used to establish whether there was a significant difference between the treatments.

Regressions were carried out in Genstat to establish whether there was a relationship between take-all assessments and yield. All these values were transformed ( $\log_{10} [X+1]$ ) before analysis to normalise the data. Simple regression with groups was used with the data sets to establish if treatments influenced the results significantly.

## 5.4 Results

### 5.4.1 Assessment of Plant Material

Both visual and molecular assessments demonstrated the presence of take-all in plant material from field plots throughout the season, with competitive PCR values ranging from 0.0002 to 507.52 pg ng<sup>-1</sup>. ANOVA completed on the data collected suggested that there was a significant difference ( $P = 0.05$ ,  $f = 0.038$ ) between the plots where Latitude with Sibutol (transformed mean = 0.8) and Baytan (transformed mean = 1.153) treatments were drilled when the TAR values for the roots of the stubble (Table 5.1) were compared. The same ANOVA calculation demonstrated a significant difference ( $P = 0.05$ ,  $f = 0.038$ ) between the plots where the Jockey (transformed mean = 0.901) and Baytan (transformed mean = 1.153) treatments were drilled. The plots where the Sibutol treated seed was drilled was not significantly different to the others. When the other data sets were subjected to ANOVA no significant relationships were observed.

Table 5.1: TAR values for the roots of the stubble collected.

Treatment	Log <sub>10</sub> stubble root TAR value (untransformed value)
Baytan	1.153 (14.32)
Jockey	0.901 (8.38)
Latitude+Sibutol	0.800 (6.52)
Sibutol	0.933 (9.58)

s.e.d. = 0.1012, l.s.d. = 0.2290, cv% = 15.1

As there was a treatment effect between the stubble root TAR values simple regressions with group were used establish how treatment effected the correlation of various data sets. In all correlations the variates were normally distributed. The only correlation where the explanatory variate was highly significant ( $f = <0.001$ ,  $P = 0.05$ ) in

the accumulated ANOVA was between the DNA concentration of roots at GS 39 and DNA concentration of stem at GS 69. The variance accounted for by the DNA concentration of roots at GS 39 was 17.6%. When the treatment was added to the explanatory variate 46% of the variability was accounted for. Treatment had a significant effect ( $f < 0.001$ ,  $P = 0.05$ ) on the response variate.

The only regression in which the explanatory variate accounted for more than 50% of the variability was when the DNA concentration of stubble stems was correlated with the TAR value of roots at GS 30 (Figure 5.1). The variability accounted for by the DNA concentration of the stem at stubble was 56.8% ( $f = 0.003$ ). In the accumulated ANOVA the DNA concentration plus treatment accounted for 67.2% of the variability but treatment was not significant ( $f = 0.403$ ).

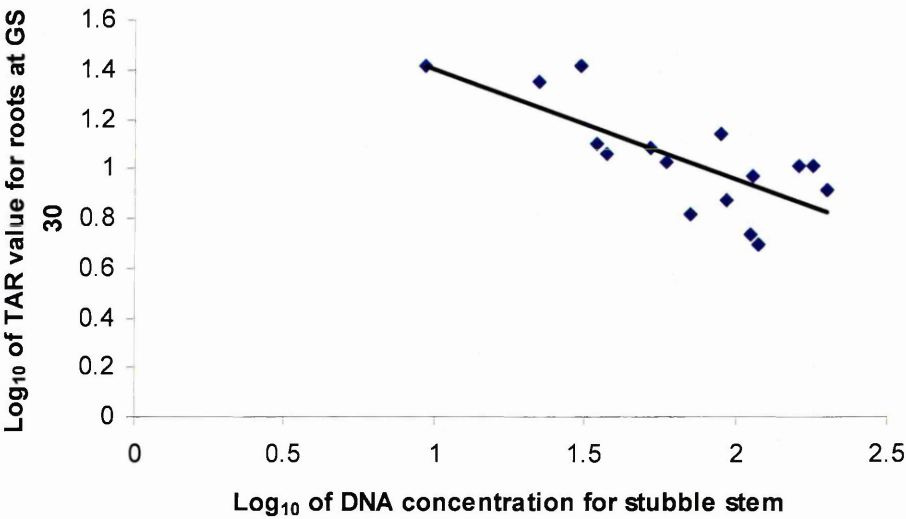


Figure 5.1: Correlation of Log<sub>10</sub> of the DNA concentration for stems at stubble and Log<sub>10</sub> of TAR value for the roots at GS 30 (variance accounted for by explanatory variate = 56.8,  $f = 0.003$ , standard error = 0.148).

A number of other regressions showed the explanatory variate having a significant effect on the TAR value for GS 69 roots, as the response variate (Table 5.2). Only when

the DNA concentration for roots at GS 39, and the DNA concentration for roots at GS 69 was correlated was the treatment effect significant (0.037 and 0.024, respectively).

Table 5.2: Regression analysis completed with the TAR values for GS 69 roots (response variate) and various explanatory variates.

Explanatory variate	Percentage variance accounted for	f value at P = 0.05	Variance accounted for when treatment was added	f value at P = 0.05	Standard error of observation
Stubble roots TAR	-33.3	0.018	57.0	0.183	0.094
GS 39 roots DNA	28.6	0.015	70.1	0.037	0.084
GS 39 roots TAR	30.9	0.018	62.4	0.094	0.090
GS 69 roots DNA	43.0	0.002	75.2	0.024	0.068

There was a negative relationship between the take-all DNA concentration of stubble stems and the TAR value of roots stubble. Both DNA concentration (-26.2%,  $f = 0.015$ ) and DNA concentration plus treatment (65.9%,  $f = 0.035$ ) had a significant effect on the TAR value. When the DNA concentration of stubble stems was correlated with the DNA concentration of roots at GS 39, the stubble DNA had a significant effect on the GS 39 DNA (29.2%,  $f = 0.034$ ). In this accumulated ANOVA treatment was not significant. Treatment was also not significant ( $f = 0.516$ ) when the TAR value for roots at GS 30 was correlated with the DNA concentration of roots at GS 39. It accounted for 39% of the variability where as the TAR value for roots at GS 30 accounted for 30.6% of the variability in the response variate and was significant ( $f = 0.017$ ).

5.4.2 Harvest

With the earlier ANOVA demonstrating that treatment had an effect on the stubble data the yield data was analysed in simple regressions with groups, with other explanatory variates to establish the effect of treatment on these correlations. Four accumulated ANOVAs completed with these regressions demonstrated the explanatory variate or the explanatory plus treatment having a significant effect on the yield. One regression with the DNA concentration of stems at stubble was positive. The addition of treatment in the accumulated ANOVA only resulted in a significant result when the DNA concentration of roots at stubble was the explanatory variate (Table 5.3).

Table 5.3: Regression analysis completed with yield in tonnes ha<sup>-1</sup> (response variate) and various explanatory variates.

Explanatory variate	Percentage variance accounted for	f value at P = 0.05	Variance accounted for when treatment was added	f value at P = 0.05	Standard error of observation
Stubble roots DNA	14.1	0.030	66.1	0.007	0.865
Stubble stem DNA	30.2	0.033	44.9	0.414	1.300
Stubble roots TAR	32.4	0.008	46.7	0.220	0.978
GS 39 roots DNA	32.9	0.031	44.8	0.516	1.330
GS 69 roots TAR	27.5	0.050	52.8	0.256	1.380

As there was a significant difference between treatments when the TAR values of roots from the stubble were compared, the stubble DNA concentrations and TAR values where inserted in ANOVA calculations as covariates to establish their significance. This analysis showed that the stubble root take-all DNA had a significant effect on yield (f =

0.006) (Table 5.4). Latitude resulted in a significantly higher yield ( $f = 0.001$ ,  $LSD = 1.066$ ) than all the other treatments, when stubble root take-all DNA was used as a covariate. This is a yield increase of  $3 \text{ t ha}^{-1}$  (30%) compared to Sibutol alone. The other stubble data sets were either not parallel, so not suitable for statistically analysis, or did not significantly affect any data sets collected later in the season. When the specific weight and thousand grain weight were subjected to ANOVA with the stubble data sets as covariates the data sets were not parallel, consequently they were not suitable for statistically analysis.

Table 5.4: Predicted mean for yield (tonnes/ha) (with the mean in the parentheses), specific weight and 1000 grain weight when the concentration of pathogen DNA for stubble roots is used as a covariate.

Treatment	Yield (tonnes/ha)	Specific weight	1000 grain weight (g)
Baytan	6.36 (6.72)	72.15	55.04
Jockey	6.67 (6.87)	72.84	53.57
Latitude+Sibutol	9.61 (8.64)	72.14	52.12
Sibutol	6.67 (7.04)	71.13	52.09
$f(\text{treatment}) = 0.001$ , $f(\text{covariate}) = 0.006$ , $LSD = 1.066$ , $cv\% = 7.8$			



## 5.5 Discussion

The ANOVA that was completed on the take-all values of the stubble showed that the previous crop was affected by take-all in different areas to varying degrees. The fact that there is a significant difference in the amount of take-all between different parts of the field supports the theory that this disease does not occur uniformly across a field.

The aim of having long thin plots was that they would reduce the variability seen within take-all experiments due to the heterogeneous nature of the disease. With the plots having a significantly different amounts of inoculum before the treatments were applied it appears that these type of plots were not effective in reducing the variability. Another experimental method which is thought to remove this variability is a split plot design (Bateman and Hornby 1999; Bateman *et al.*, 1994). This involves having blocks of small paired, plots which are replicated across a field or fields. The problem with this type of design is that it is difficult to manage. Small plots require specialist equipment for drilling, treating and harvesting. Large plots used in this experiment allow the plots to be incorporated into standard farming practices using standard equipment.

When the covariate ANOVA with stubble root take-all DNA was used to compare the yield with different treatments it showed that both the seed treatment and the covariate had a significant effect on the yield. This indicates that DNA concentration of roots in the stubble did influence yield and may be useful in experiments to take into account the effect of inoculum on the yield of a crop within a field. Using the DNA concentration of roots in the stubble as a covariate resulted in a *f* value of increased significance (from 0.034 to 0.001) for the treatments. The percentage cumulative variance was reduced from 12.2 to 7.8. Using the covariate analysis appears to strengthen the conclusion that Latitude resulted in significantly greater yield than the other treatments.

Both Latitude and Jockey treatments have been shown to have an effect on yield or disease severity. Latitude (applied with Beret Gold) when compared to Baytan and Beret Gold (fludioxonil) in plot experiments on a variety of soils demonstrated a reduction in disease severity (Knight, 2002). These differences in disease tend to lessen as the season progresses but the yield is still greater in plots where the seed was treated with Latitude. It was indicated that where the disease severity is high the yield loss is reduced by the application of Latitude, as demonstrated in these experiments. Early work using Latitude indicated that it delayed early infection of the roots, limiting the spread of disease from plant to plant. It was less effective at preventing the spread of disease within the plant. Early epidemics of take-all were reduced to a greater extent than late epidemics (Schoeny and Lucas, 1999). Schoeny *et al.* (2001) highlighted the fact that a greater recovery of yield was seen in severe epidemics than in less severe ones, suggesting that any differences seen in this experiment would be increased if the epidemic had been more severe. It has been proposed that Latitude increases effective root density consequently increasing water and nitrogen uptake. Seed treatment increased the available assimilates at grain fill by reducing the disease severity, and hence there was no reduction in flow in the vessels. This was observed in an increase in specific and 1000 grain weights (Spink *et al.*, 2002). These results were not reflected in this experiment.

Fluquinconazole, a component of Jockey, was discovered to have action against a number of foliar diseases in 1992 (Russell *et al.*). Further work suggested that it resulted in at least a 20% recovery in the yield (Wenz *et al.*, 1988; Bardsley *et al.*, 2000). It has been seen to increase thousand grain weights (Lochel *et al.*, 1998). Dawson and Bateman (2001) observed Jockey decreasing disease and increasing yields in the second and third year of a wheat crop. It was noted that the treatment had no effect on the fungal communities in the soil. When the treatment was not applied the amount of disease increased indicating that take-all decline had not been initiated. Bateman *et al.* (2003b) suggest that it is

economically viable to apply Jockey to a second or third wheat crop to prevent severe take-all epidemics. Testing of Jockey F (fluquinconazole only) and Latitude as seed treatments on field crops of wheat indicated that Latitude was as effective as Jockey F (fluquinconazole only) (Bateman *et al.*, 2004). The experiment in this chapter suggests that Latitude is more effective at controlling take-all as demonstrated by the significant increase in yield. In fact, in this experiment Jockey treatment resulted in a lower yield than the plots treated with Sibutol. As this is only one experiment it would have to be repeated before conclusions could be drawn.

The results from this experiment suggest that Baytan does not result in an increase in yield, due to a decrease in take-all symptoms as seen by Werker and Gilligan (1990). They observed that any control by Baytan was inconsistent; it was most effective when used on early sown crops. Baytan was observed to reduce the number of diseased roots per plant rather than number of diseased plants in a plot. Other work showed triadimenol, a component of Baytan, to be effective against take-all when it was applied evenly to soil in pots (Bateman *et al.*, 1990). The reduction in disease symptoms and yield benefit seen with Baytan was not as great as that observed when Latitude was applied with Beret Gold (Knight, 2002). This would support the results of this study.

All of the regressions attempted do not demonstrate a consistent picture of the relationship between TAR values and DNA concentrations. Some regressions appear to contradict each other. There is a negative correlation between the DNA concentration of stubble stems and the TAR value of roots at GS 30 but, when the DNA concentration for the roots at GS 39 and roots at GS 69 are correlated the regression is positive. Although as with Chapter Four, the correlation of the TAR values for stubble stem and roots resulted in a positive percentage variance. Discovering the reason why these data do not result in any strong relationship is difficult. The same cultivar, from the same seed batch, was used for all plots. Consequently, the host resistance should be the same for each plot, although the

treating of the seed may have influenced host resistance. Less than half of the regressions in the results indicated that treatment had a significantly greater effect on the response variate, and only one is highly significant. This is demonstrated by treatment not being significant in the accumulated ANOVA. Consequently treatment appears to have an inconsistent effect on the various data. Also, there is no evidence to indicate whether the *Ggt* populations varied across the field or not. Different populations of *Ggt* may demonstrate varying pathogenicity.

The regression between pathogen DNA concentration in the roots of stubble and yield suggests a relationship, where the amount of yield decreases as the amount of disease increases. This is the only regression where the treatment has a highly significant effect. It would appear that in this case the treatment has a greater effect on the yield than the pathogen DNA at stubble does. These results support the observation that the inoculum was not spread evenly across the field, a classic trait of take-all. This regression is in contrast to the fact that a positive correlation was observed between stem DNA at the same time and yield. Other negative relationships were seen when the yield was correlated with DNA from the roots at GS 39 and the TAR value for the roots at stubble and at GS 69. In these regressions the fact that the treatment was not significant, indicates that the treatment did not influence these regressions. These results are confusing as they present two different views where an increasing amount of disease, correlates with decreasing and increasing yield. Previous evidence has suggested that take-all, in particular when epidemics are severe, reduces yield (Polley and Clarkson, 1980). In fact it would appear to be illogical for a plant pathogen to cause disease on a crop and increases the yield. The results do not allow any suggestions or theories to be formulated.

This experiment was small in that it only included four replicates in one field. For conclusions to be drawn and to clarify confusing results, more experiments with more

replicates in a variety of different fields would need to be completed. Even so it possible to suggest that;

- Latitude seed treatment can result in a greater yield when compared to Baytan, Jockey and Sibutol seed treatments
- the DNA concentration of take-all pathogens in the roots of stubble may influence the amount of yield obtained from a subsequent crop of winter wheat
- it may be possible to use the DNA concentration of take-all pathogens in roots of stubble as an indicator of take-all inoculum prior to completing field experiments

The results in this study support the fact that take-all is a heterogeneous pathogen. They also suggest that long thin plots may not reduce the overall variability observed within take-all field experiments. In addition, if the take-all DNA is measured in the roots of stubble of the previous crop it can be used to demonstrate differences in the inoculum of plots, and can be used as a covariate in the analysis of treatments effects to take account of inoculum. The competitive PCR assay proved to be valuable in establishing the inoculum concentration of take-all.

# **Chapter Six**

## **General Discussion**

Take-all is a severe disease of wheat causing significant losses to yield (Rosser and Chambers, 1968; Polley and Clarkson, 1980). The causative agent of this disease, *Ggt*, has been found to survive in the debris of plants from previous host crops. From this it is able to grow towards the roots of any subsequent host crops, causing black lesions on the stems and roots (Brown and Hornby, 1971). The mycelium blocks the vessels in the stele affecting the supply of water and nutrients to the rest of the plant (Liu *et al.*, 2000). Consequently, it reduces grain size and promotes the early ripening of the seed, and hence reduces yield (Clarkson and Polley, 1981).

The extraction of organic matter from soil and subsequently the DNA has successfully been achieved in the testing from *Ggt* by Herdina *et al.* (1996), but they used a slot-blot hybridisation assay to quantify the *Ggt* which is not as sensitive to inhibition as a PCR assay. As the aim of this project was to use a competitive PCR assay in the test it is perhaps not surprising that initial studies presented problems. Soil contains humic acids which inhibit the PCR (Tsai and Olson, 1992a). Theories about how they do this are associated with the chelation of humic acids with the DNA polymerase and the magnesium ions although conflicting results are obtained (Tsai and Olson, 1992b). A number of authors (for example, Herdina *et al.*, 1997; Miller *et al.*, 1999; Griffiths *et al.*, 2000) claim to have successfully removed the humic acids from the solution containing the DNA, but they often require lengthy and expensive purification processes (for example, Herdina *et al.*, 1996; Miller, 2001). Spectrophotometry of the DNA samples tested showed that even within a method the purity of samples varied.

Studies have shown that spectrophotometry, a standard laboratory technique for assessing DNA quality can be unreliable when used with DNA extracted from soil. Humic acids are known to absorb light at one of the wavelengths (260 nm) used to measure DNA (Cullen and Hirsch, 1998); as a result this author designed an inhibition assay to establish the contamination present in samples. The inhibition assay in this study demonstrated that

repeated dilutions are needed to enable the DNA extracted from soil organic matter to be amplified. It suggested that the CTAB method (method 3) was the most effective of all the methods tested at removing inhibition from soil organic matter. Samples from this method required less dilution before they could be amplified. When testing soil for a pathogen a negative result could be due to the presence of inhibition rather than the absence of the pathogen. Consequently it would be necessary to test samples for inhibition. This would have to be completed for all samples as the inhibition appears to vary with samples taken from a single field or from different extractions of the same sample. Any increase in the processing time of a sample increases the time before a farmer would have the information making it less likely to be useful. Inhibition would make an assay less reliable and, with plants stem base and roots being successfully used with other plant pathogens (for example, Nicholson *et al.*, 1997; Doohan *et al.*, 1999) this author felt more confident in using plants as the medium to extract DNA from. The results in Chapter Four suggest that it is possible to obtain DNA that is of a quality to use in a PCR assay from stubble and from roots, as well as stem bases of growing plants.

A slot hybridization assay has been developed to quantify the presence of the fungus in soil (Herdina *et al.*, 1996), correlating it with the amount of disease observed on the plants (Herdina *et al.*, 1997). The authors believed that the assay could be used to predict disease risk, but this work was completed on soil in Australia where the epidemiology of take-all may be different to the UK (Hornby, 1998a). The probe was also developed using isolates from Australia. It was not tested on isolates or soil found in the UK. Harvey *et al.* (2001) highlighted the fact that take-all populations are often geographically isolated and any genetic transfer is limited as sexual reproduction is limited. As the UK and Australia are islands great distances apart the genetic transfer between populations would have to be by plant debris carried from one country to the next. This seems unlikely particularly as only the grain is transported. Therefore, it would be unlikely that there could be any mixing of the populations and the populations in the two



countries could be genetically different, meaning any assay designed in Australia maybe unreliable in the UK. This maybe true of any probes or primers for PCR assays designed in other countries, although there is no direct evidence to prove this. PCR primer pairs have been designed to amplify *G. graminis* varieties. The primers AVE/R and Ggg, Gga and Ggt, (Rachdawong *et al.*, 2002) and the pair NS5: GGT-RP (Fouly and Wilkinson, 2000) were designed and tested on isolates from the USA. A difference between the DNA of the USA and UK populations of *G. graminis* would explain why this primers did not amplify as expected. However this does not explain the results obtained in this study with the primer pairs KS1:KS2 (Ward, 1995) and pGgt1:pGt2 (Bryan *et al.*, 1995). In addition the primer pair pGgt1:pGt2 did not amplify the isolates as expected as amplification resulted in multiple bands. If there is more than one target band expected in competitive PCR the efficiency of the amplication of the bands, including the internal standard, is affected. Any concentration calculations based on these bands' intensities would be inaccurate. The unexpected results may also be due to differences in the PCR assay. Primers that are based on single base differences are highly dependant on PCR conditions for stringency.

As none of the published primers amplified as expected the HAGG primers were developed. They amplified all the *Ggt* and *Gga* isolates and all of the *G. graminis*-like isolates grown from the blackened lesions of wheat roots suspected of having take-all. The HAGG competitive PCR assay worked with the plant material that was collected from commercial wheat fields, quantifying the presence of the fungus. Unfortunately, the only large, significant correlation was between the visual disease assessments of stubble. A possible reason for this could be that the assay is too sensitive or not sensitive enough resulting in the quantification of the fungus that is not representative of the visual symptoms assessed. It may be that when the plants were assessed for visual symptoms there was an over or under estimation of the severity of the disease. Using commercial fields could have consequences on the ability to correlate visual and molecular assessments. Any treatment, for example with fungicides or fertiliser, may affect the

relationship. If they are applied, host resistance or fungal pathogenicity, may be affected so that although the fungus may be present the plant is able to compensate. In most of the ANOVAs when field was used in addition to the explanatory variate in simple regressions with groups it had a significant effect on the response variate. This indicates that field effects can influence the progress of disease within a field and can be the reason for the different levels of take-all observed in different fields. Fields effects would include soil type, and whether different cultivars or sowing dates were used. The application of fungicides may also influence the growth of the pathogen.

The above explanations only describe why there is variability within the data, particularly between fields but it does not explain why there is no correlation between data sets, and as yet this author can not explain this. As there appeared to be few strong correlations, any modelling to predict the disease risk, at this stage would not be possible.

Fungicides, Baytan (Werker and Gilligan, 1990), Jockey (Bateman *et al.*, 2003a) and Latitude (Knight, 2003) have been shown in the past to control take-all and consequently to increase yields. In Chapter Five, Latitude resulted in the greatest yield whereas the yield of Baytan treated plots was the least. In contrast to previous studies (Bardsley *et al.*, 2000) the Jockey treatment did not result in a yield as great as the control plots, in this case Sibutol treated. This treatment is not thought to have any action against take-all. Using the covariate analysis allowed the incorporation of the stubble data into the analysis of the treatments later in the year. Consequently, it was possible to determine that Latitude resulted in a significantly greater yield than the other treatments when the take-all DNA concentration of the stubble roots was used as a covariate.

The fact that DNA concentration of stubble roots influenced the yield shows that using the HAGG competitive PCR assay to measure this variable would be useful in the determination of the effect of inoculum on a field trial. In the commercial fields, field accounted for a greater proportion of the variability than DNA concentration, or visual symptoms. Consequently, DNA concentration of stubble roots would not be of value in

these fields unless the other variables within 'field' were known and could be measured. However, when field trials are completed in the same field it would be of use as these variables are removed.

## 6.1 Further Work

The DNA extractions completed in this study demonstrate the difficulty in extracting DNA from soil that does not contain PCR inhibitors. Further studies of the interaction between soil inhibitors and DNA could elude to a method that is reliable and quick. Consequently, enabling the amplification of DNA from a range of soil borne pathogens and pests from DNA extracted from soil or soil organic matter, including using this take-all competitive PCR.

This study presents a new method of quantifying take-all but as it could not find a correlation between take-all symptoms or DNA concentration in the field and yield more work is required to establish the cause of this. It is likely that climate and soil conditions, and probably any fertiliser or fungicide are influential in this disease dynamic, but they do not explain the lack of correlation between the DNA concentration of the pathogen and take-all symptoms. A further study could include data of this nature and in concert with the competitive PCR assay enable a fuller understanding of the factors affecting take-all severity and yield loss in a season. Then, it may be possible to construct a model.

A reason for using PCR rather than DNA hybridisation is that it is an easier and more sensitive method of identification. A new technology, real time PCR is now available and may offer an even easier and therefore quicker test. To do this it would be necessary to construct primers that produced a product of a smaller size, approximately 100 base pairs. This could be achieved by moving the location of the reverse primer so it binds to a different section of the internal transcribed spacer nearer to the forward primer.

Previously published primers did not appear to work, which may be due to changes in the population of *Ggt*, so monitoring of the populations may be necessary. Sequencing of the 18S rRNA gene, the 5.8S gene and internal transcribed spacers has in the past shown how related the varieties are and may demonstrate any sequence changes that may effect the attachment of primers (Bryan *et al.*, 1995). Just prior to the submission of this thesis another sequence of DNA was added to the database used to design the HAGG, highlighting the ever changing situation with regards the sequence information available for DNA of *Ggt* populations (Freeman *et al.*, 2005).

Pathogen DNA concentrations of stubble roots was observed to effect the yield obtained in the field trial in this study. Its use in further field trials would enable the inoculum present in the field to be taken into account in later assessments. It would also, indicated the distribution of the disease across a field.

The fact that Jockey did not perform as well as expected demonstrates the complexities of this disease. Further long term studies would be needed to establish the significance of using Latitude in contrast to Jockey, and which one of the treatments is the best at controlling the disease and increasing the yield obtained. It may be the case that each chemical is more suitable for certain agronomic conditions.

## References

Amein, T. and Weber, Z. 2002. Seed treatment with strains of *Pseudomonas fluorescens* as potential biocontrol agents of wheat take-all. *Journal of Plant Disease and Protection*. **109**, 655 – 651.

Annamalai P., Ishii, H, Lalithakumari, D., and Revathi, R. 1995. Polymerase chain reaction and its applications in fungal disease diagnosis. *Journal of Plant Diseases and Protection*. **102**, 91-104.

Bailey, D. J. and Gilligan, C. A. 1999. Dynamics of primary and secondary infection in take-all epidemics. *Phytopathology*. **89**, 84 – 91.

Bailey, D. J. and Gilligan, C. A. 2004. Modelling and analysis of disease - Induced host growth in the epidemiology of take-all. *Phytopathology*. **94**, 535 – 540.

Ballinger, D. J. and Kollmorgen, J. F. 1986. Control of take-all of wheat in the field with benzimidazole and triazole fungicides applied at seeding. *Plant Pathology*. **35**, 67 – 73.

Bardsley, E. S., Davies, P. H. and Hopkinson, D. 2000. Fluquinconazole seed treatment as a management tool for the control of foliar disease and optimization of yield in winter wheat. **In: The BCPC Conference – Pests and Diseases 2000**. **3**, 877 – 882. Farnham, Surrey: British Crop Protection Council.

Barnett, S. J., Singleton, I. and Ryder, M. 1999. Spatial variation in populations of *Pseudomonas corrugata* 2140 and pseudomonads on take-all diseased and healthy root systems of wheat. *Soil Biology and Biochemistry*. **31**, 633 – 636.

Bateman, G. L. 1984. Soil-applied fungicides for controlling take-all in field experiments with winter wheat. *Annals of Applied Biology*. **104**, 459 – 465.

Bateman, G. L. and Hornby, D. 1999. Comparison of natural and artificial epidemics of take-all in sequences of winter wheat crops. *Annals of Applied Biology*. **135**, 55 – 571.

Bateman, G. L. and Kwasna, H. 1999. Effects of number of winter wheat crops grown successively on fungal communities on wheat roots. *Applied Soil Ecology*. **13**, 271 – 282.

Bateman, G. L., Nicholls, P. H. and Chamberlain, K. 1990. The effectiveness of eleven sterol biosynthesis-inhibiting fungicides against the take-all fungus, *Gaeumannomyces graminis* var. *tritici*, in relation to their physical properties. *Pesticide Science*. **29**, 109 - 122.

Bateman, G. L., Ward, E. and Antoniwi, J. F. 1992. Identification of *Gaeumannomyces graminis* var. *tritici* and *G. graminis* var. *avenae* using a DNA probe and non-molecular methods. *Mycological Research*. **96**, 737 – 742.

Bateman, G. L., Hornby, D., Payne, R. W. and Nicholls, P. H. 1994. Evaluation of fungicides applied to soil to control naturally-occurring take-all using a balanced-incomplete-block design and very small plots. *Annals of Applied Biology*. **124**, 241 – 251.

Bateman, G. L., Edwards, S. G., Marshall, J., Morgan, L. W., Nicholson, P., Nuttall, M., Parry, D. W., Scrancher, M. and Turner, A. S. 2000. Effects of cultivar and fungicides on stem-base pathogens, determined by quantitative PCR, and on diseases and yield of wheat. *Annals of Applied Biology*. **137**, 213 – 221.

Bateman, G. L., Freeman, J., Gutteridge, R. J., Jenkyn, J. F. and Ward, E. 2003a. Strategies for fungicidal control of take-all. *Home-Grown Cereals Authority Project. Report No. 309*.

Bateman, G. L., Jenkyn, J. F. and Gutteridge, R. J. 2003b. Optimising the benefits of fluquinconazole seed treatment in sequences of winter wheat crops. **In:** *The BCPC International Congress - Crop Science and Technology 2003*. **2**, 979 – 984. Farnham, Surrey. British Crop Protection Council.

Bateman, G. L., Gutteridge, R. J. and Jenkyn, J. F. 2004. Take-all and grain yields in sequences of winter wheat crops testing fluquinconazole seed treatment applied in different combinations of years. *Annals of Applied Biology*. **145**, 317 – 330.

Beale, R. E., Lavier, B., Becker, J., Leaper, D. and Stafford, N. B. 1998a. A model for the prediction of yield loss in wheat due to take-all disease caused by *Gaeumannomyces graminis* var. *tritici*. **In:** *The 1998 Brighton Conference - Pests and Diseases*. **3**, 1035 – 1040. Farnham, Surrey: British Crop Protection Council.

Beale, R. E., Phillion, D. P., Headrick, J. M., O'Reilly, P. and Cox, J. 1998b. MON: 65500: A unique fungicide for the control of take-all in wheat. **In:** *The 1998 Brighton Conference - Pests and Diseases*. **2**, 343 - 350. Farnham, Surrey: British Crop Protection Council.

Becker, J., Lavier, B. and Leaper, D. J. 1998. Take-all (*Gaeumannomyces graminis* var. *tritici*) infestation survey for Germany, France and the UK, 1996 – 1997. **In:** *The 1998 Brighton Conference – Pests and Diseases*. **1**, 77 – 82. Brighton, Surrey. British Crop Protection Council.



- Braid, M. D., Daniels, L. M. and Kitts, C. L. 2003. Removal of PCR inhibitors from soil DNA by chemical flocculation. *Journal of Microbiological Methods*. **52**, 389 – 393.
- Brown, M. E. 1981. Microbiology of roots infected with the take-all fungus (*Gaeumannomyces graminis* var. *tritici*) in phased sequences of winter wheat. *Soil Biology and Biochemistry*. **13**, 285 -291.
- Brown, M. E. and Hornby, D. 1971. Behaviour of *Ophiobolus graminis* on slides buried in soil in the presence or absence of wheat seedlings. *Transactions of the British Mycological Society*. **56**, 95 – 103.
- Bryan, G. T., Daniels, M. J, Osbourn, A. E. 1995. Comparison of fungi within the *Gaeumannomyces* - *Phialophora* complex by analysis of ribosomal DNA sequences. *Applied and Environmental Microbiology*. **61**, 681-689.
- Bryan, G. T., Labourdette, E., Melton, R. E., Nicholson, P. Daniels, M. J. and Osbourn, A. E. 1999. DNA polymorphism and host range in the take-all fungus, *Gaeumannomyces graminis*. *Mycological Research*. **103**, 319 – 327.
- Burgmann, H., Pesaro, M., Widmer, F. and Zeyer, J. 2001. A strategy for optimizing quality and quantity of DNA extracted from soil. *Journal of Microbiological Methods*. **45**, 7 – 20.
- Carter, J. P., Spink, J., Cannon, P. F., Daniels, M. J. and Osbourn, A. E. 1999. Isolation, characterization, and avenacin sensitivity of a diverse collection of cereal-root-colonizing fungi. *Applied and Environmental Microbiology*. **65**, 3364 – 3372.

Chambers, S. C. and Flentje, N. T. 1967. Studies on oat-attacking and wheat-attacking isolates of *Ophiobolus graminis* in Australia. *Australian Journal of Biological Science*. **20**, 927 – 940.

Christensen, N. W., Powelson, R. L. and Brett, M. 1987. Epidemiology of wheat take-all as influenced by soil pH and temporal changes in inorganic soil N. *Plant and Soil*. **98**, 221 – 230.

Clarkson, J. D. S. and Polley, R. W. 1981. Assessment of losses caused by stem-base and root disease in cereals. **In: Proceedings of 1981 Brighton Crop Protection Conference – Pests and Diseases**. **1**, 223 – 231. Farnham, Surrey. British Crop Protection Council.

Colbach, N., Lucas, P. and Meynard, J-M. 1997. Influence of crop management on take-all development and disease cycles on winter wheat. *Phytopathology*. **87**, 26 – 32.

Cook, R. J. 1981. The influence of rotation crops on take-all decline phenomenon. *Phytopathology*. **71**, 189 – 192.

Conventry, D. R., Brooke, H. D., Kollmorgen, J. F. and Ballinger, D. J. 1989. Increases in wheat yield on limed soil after reduction of take-all by fungicide application and crop rotation. *Australian Journal of Experimental Agriculture*. **29**, 85 - 89.

Cotterill, P. J. and Sivasithamparam, K. 1987a. Take-all inoculum: its occurrence and distribution within a wheat crop and a fallow. *Soil Biology and Biochemistry*. **19**, 221 – 222.

- Cotterill, P. J. and Sivasithamparam, K. 1987b. Intermittent wetting of soils at high temperature reduces survival of the take-all fungus. *Plant and Soil*. **103**, 289 – 291.
- Cotterill, P. J. and Sivasithamparam, K. 1988a. The effect of tillage practices on distribution, size, infectivity and propagule number of the take-all fungus (*Gaeumannomyces graminis* var. *tritici*). *Soil and Tillage Research*. **11**, 183 – 195.
- Cotterill, P. J. and Sivasithamparam, K. 1988b. Inoculum of the take-all fungus in rotations of wheat and pasture: relationships to disease and yield of wheat. *Transactions of the British Mycological Society*. **91**, 63 – 72.
- Cotterill, P. J. and Sivasithamparam, K. 1988c. Effect of sowing date on take-all of wheat in Western Australia. *Phytophylactica*. **20**, 11-14.
- Cunningham, P. C. 1981. Isolation and Culture. **In:** M. J. C. Asher and P. J. Shipton. *eds. Biology and Control of Take-all*. London. Academic Press. pp. 103-124.
- Cullen, D. W. and Hirsch, P. R. 1998. Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biology and Biochemistry*. **30**, 983 – 993.
- Dawson, W. A. J. M. and Bateman, G. L. 2001. Fungal communities on roots of wheat and barley and effects of seed treatments containing fluquinconazole applied to control take-all. *Plant Pathology*. **50**, 75 – 82.
- Deacon, J. W. 1974. Further studies on *Phialophora radicola* and *Gaeumannomyces graminis* on roots and stem bases of grasses and cereals. *Transactions of the British Mycological Society*. **63**, 307-327.

- Delp, B. R., Stowell, L. J. and Marois, J. J. 1986. Evaluation of field sampling technology for establishing disease incidence. *Phytopathology*. **76**, 1299 – 1305.
- Dewan, M. M. and Sivasithamparam, K. 1990. Differences in the pathogenicity of isolates of the take-all fungus from roots of wheat and ryegrass. *Soil Biology and Biochemistry*. **22**, 119 - 122.
- Doohan, F. M., Parry, D. W., Jenkinson, P., Nicholson, P. 1998. The use of species-specific PCR-based assays to analyse *Fusarium* ear blight of wheat. *Plant Pathology*. **47**, 197-205.
- Doohan, F. M., Parry, D. W. and Nicholson, P. 1999. *Fusarium* ear blight of wheat: the use of quantitative PCR and visual disease assessment in studies of disease control. *Plant Pathology*. **48**, 209 – 217.
- Duffy, B. K. and Weller, D. M. 1994. A semiselective and diagnostic medium for *Gaeumannomyces graminis* var. *tritici*. *Phytopathology*. **84**, 1407 – 1417.
- Edwards, S. G., Pirgozliev, S. R., Hare, M. C. and Jenkinson, P. 2001. Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against *Fusarium* head blight of winter wheat. *Applied and Environmental Microbiology*. **67**, 1575- 1580
- Epstein, L., Kaur, S., Goins, T., Kwon, Y. H. and Henson, J. M. 1994. Production of hyphopodia by wild-type and three transformants of *Gaeumannomyces graminis* var. *graminis*. *Mycologia*. **86**, 72 – 81.

Faggian, R., Parsons, S., Porter, I. J., Lawrie, A. C. 2001. Detection of clubroot in Australia: progress towards a reliable molecular diagnostic assay. **In:** I. J. Porter *et al.* Eds. *Proceedings of the Second Australasian Soilborne Diseases Symposium*. Lorne 5-8 March 2001. [On-line] Available from <http://www.nre.vic.gov.au/agvic/ihd/publications/asds-fagian.pdf> [Accessed June 2002]

Fellows, H. 1941. Effect of certain environmental conditions on the prevalence of *Ophiobolus graminis* in the soil. *Journal of Agricultural Research*. **63**, 715 – 726.

Filion, M., St-Arnaud, M. and Jabaji-Hare, S. H. 2003. Direct quantification of fungal DNA from soil substrate using real-time PCR. *Journal of Microbiological Methods*. **53**, 67 – 76.

Fouly, H. M. and Wilkinson, H. T. 2000. Detection of *Gaeumannomyces graminis* varieties using polymerase chain reaction with variety-specific primers. *Plant Disease*. **84**, 947-951.

Fouly, H. M., Wilkinson, H. T. and Domier, L. L. 1996. Use of random amplified polymorphic DNA (RAPD) for identification of *Gaeumannomyces* species. *Soil Biology and Biochemistry*. **28**, 703-710.

Fouly, H. M., Wilkinson, H. T. and Chen, W. 1997. Restriction analysis of internal transcribed spacers and the small subunit gene of ribosomal DNA among four *Gaeumannomyces* species. *Mycologia*. **89**, 590 – 597.

Frederick, B. A., Caesar-Tonthat, T-C., Wheeler, M. H., Sheehan, K. B., Edens, W. A. and Henson, J. M. 1999. Isolation and characterisation of *Gaeumannomyces graminis* var. *graminis* melanin mutants. *Mycological Research*. **103**, 99 – 110.

Freeman, J. and Ward, E. 2004. *Gaeumannomyces graminis*, the take-all fungus and its relatives. *Molecular Plant Pathology*. **5**, 235 – 252.

Gams, W. and Domsch, K. H. 1969. The spatial and seasonal distribution of microscopic fungi in arable soils. *Transactions of the British Mycological Society*. **52**, 2, 301 – 308.

Garcia, C. and Mathre, D. E. 1987. Factors affecting control of take-all of spring wheat by seed treatment with sterol biosynthesis-inhibiting fungicides. *Plant Disease*. **71**, 743 – 746.

Garrett, S. D. 1934. Factors affecting the severity of take-all. 3. The climatic factor. *Journal of Agriculture*. **37**, 976 – 983.

Garrett, S. D. 1940. Soil conditions and the take-all disease of wheat. V. Further experiments on the survival of *Ophiobolus graminis* in infected wheat stubble buried in the soil. *Annals of Applied Biology*. **27**, 199 – 204.

Garrett, S. D. 1944. Soil conditions and the take-all disease of wheat. VIII. Further experiments on the survival of *Ophiobolus graminis* in infected wheat stubble. *Annals of Applied Biology*. **31**, 186 – 191.

Glynne, M. D. 1951. Effects of cultural treatments on wheat and on the incidence of eyespot, lodging, take-all and weeds. *Annals of Applied Biology*. **38**, 655 – 688.

- Goins, T. Q., Eden, W. A. and Henson, J. M. 2002. Heavily-melanized variants of the sexual *Gaeumannomyces graminis* var. *tritici* are non-pathogenic and indistinguishable from the asexual, *Phialophora* state. *Mycological Research*. **106**, 1179 – 1186.
- Golldack, J., Augustin, C., Lentzsch, P. and Werner, A. 2004. Pathozones of genetic subtypes of *Gaeumannomyces graminis* in cereals. *Soil Biology and Biochemistry*. **36**, 145 – 154.
- Griffiths, R. I., Wheiteley, A. S., O'Donnell, A. G. and Bailey, M. J. 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology*. **66**, 5488 – 5491.
- Gutteridge, R. J., Hornby, D., Hollins, T. W., Prew, R. D. 1993. Take-all in autumn sown wheat, barley, triticale and rye grown with high and low inputs. *Plant Pathology*. **42**, 425-431
- Hardwick, N. V., Jones, D. R. and Slough, J. E. 2001. Factors affecting diseases of winter wheat in England and Wales, 1989 – 98. *Plant Pathology*. **50**, 453 – 462.
- Harvey, P. R., Langbridge, P. and Marshall, D. R. 2001. Genetic drift and host-mediated selection cause genetic differentiation among *Gaeumannomyces graminis* populations infecting cereals in southern Australia. *Mycological Research*. **103**, 927 – 935.
- Heinz, R. A. and Platt, H. W. 2000. Improved DNA extraction method for *Verticillium* detection and quantification in large-scale studies using PCR-based techniques. *Canadian Journal of Plant Pathology*. **22**, 117 – 121.

- Heinz, R. A., Lee, S. W., Saparno, A., Nazar, R. N. and Robb, J. E. 1998. Cyclical systemic colonization in *Verticillium* infected tomato. *Physiological Molecular Plant Pathology*. **52**, 385 – 396.
- Henson, J. M. 1989. DNA probe for identification of the take-all fungus, *Gaeumannomyces graminis*. *Applied and Environmental Microbiology*. **55**, 284-288.
- Henson, J. M. and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology*. **31**, 81-109.
- Henson, J. M., Goins, T., Grey, W., Mathre, D. E. and Elliott, M. L. 1993. Use of polymerase chain reaction to detect *Gaeumannomyces graminis* DNA in plants grown in artificially and naturally infested soils. *Phytopathology*. **83**, 283-287.
- Herdina and Roget, D. K. 2000. Prediction of take-all disease risk in field soils using a rapid and quantitative DNA soil assay. *Plant and Soil*. **227**, 87-98.
- Herdina, Harvey, P. and Ophel-Keller, K. 1996. Quantification of *Gaeumannomyces graminis* var. *tritici* in infected roots and soil using slot-blot hybridisation. *Mycological Research*. **100**, 962-970.
- Herdina, Yang, H. A. and Ophel-Keller, K. 1997. Correlation of take-all disease severity and inoculum level of *Gaeumannomyces graminis* var. *tritici* using a slot-blot hybridisation assay. *Mycological Research*. **101**, 1311-1317.



- Herdina, Neate, S., Jabaji-Hare, S. and Ophel-Keller, K. 2004. Persistence of DNA of *Gaeumannomyces graminis* var. *tritici* in soil as measured by a DNA-based assay. *FEMS Microbiology Ecology*. **47**, 143 – 152.
- Holden, M. and Ashby, M. 1981. Growth on oat-seedling agar of isolates of *Gaeumannomyces graminis* and some *Phialophora* species from cereal roots. *Transactions of the British Mycological Society*. **77**, 543 – 547.
- Hollins, T. W. and Scott, P. R. 1990. Pathogenicity of *Gaeumannomyces graminis* isolates to wheat and rye seedlings. *Plant Pathology*. **39**, 269 – 273.
- Hornby, D. 1968. Methods of assessing *Ophiobolus graminis*. *Rothamsted Experimental Station Report for 1967*: 138-139.
- Hornby, D. 1975. Inoculum of the take-all fungus: nature, measurement, distribution and survival. *European Plant Pathology Organisation Bulletin*. **5**, 319 – 333.
- Hornby, D. 1998a. *Take-all Disease of Cereals: A Regional Perspective*. Wallingford. CAB International.
- Hornby, D. 1998b. Interaction between cereal husbandry and take-all: background for newer methods of controlling the disease. **In:** *The 1998 Brighton Conference – Pest and Diseases*. **1**, 67 – 76. Farnham, Surrey. British Crop Protection Council.
- Hornby, D., Slope, D. B., Gutteridge, R. J. and Sivanesan, A. 1977. *Gaeumannomyces cylindrosporus*, a new ascomycete from cereal roots. *Transactions of the British Mycological Society*. **69**, 21 – 25.

Hornby, D., Bateman, G. L., Payne, R. W., Brown, M. E., Henden, D. R. and Campbell, R. 1993. Field tests of bacteria and soil-applied fungicides as control agents for take-all in wheat. *Annals of Applied Biology*. **122**, 253 – 270.

Hornsten, L., Su, C., Osbourn, A. and Oliw, E. H. 2002. Cloning of the manganese lipooxygenase gene homology with the lipooxygenase gene family. *European Journal of Biochemistry*. **269**, 2690 – 2697.

Howeler, M., Ghiorse, W. C. and Walker, L. P. 2003. A quantitative analysis of DNA extraction and purification from compost. *Journal of Microbiological Methods*. **54**, 37 – 45.

Hughes, G. 1999. Sampling for decision making in crop loss assessment and pest management: Introduction. *Phytopathology*. **89**, 11, 1080 – 1083.

Jenkyn, J. F. and Prew, R. D. 1973. Activity of six fungicides against cereal foliar and root diseases. *Annals of Applied Biology*. **75**, 241 – 252.

Jenkyn, J. F. and Gutteridge, R. J. 2002. Effects of azoxystrobin on wheat take-all. *Home-Grown Cereals Authority Project. Report No. 285*.

Jenkyn, J. F., Bateman, G. L., Gutteridge, R. J. and Edwards, S. G. 2000. Effects of foliar sprays of azoxystrobin on take-all in wheat. *Annals of Applied Biology*. **137**, 99 – 106.

- Joseph-Horne, T., Holloman, D. W. and Heppner, C. 2000. Silthiofam interferes with mitochondrial function in 'take-all' disease of wheat. **In: *The BCPC Conference – Pests and Diseases 2000***. **3**, 883 – 888. Farnham, Surrey: British Crop Protection Council.
- Juhnke, M. E., Mathre, D. E. and Sands, D. C. 1984. A selective medium for *Gaeumannomyces graminis* var. *tritici*. *Plant Disease*. **68**, 233 – 236.
- Kabbage, M. and Bockus, W. W. 2002. Effect of placement of inoculum of *Gaeumannomyces graminis* var. *tritici* on severity of take-all winter wheat. *Plant Disease*. **86**, 298 – 303.
- Klerks, M. M., Zijlstra, C. and van Bruggen, A. H.C. 2004. Comparison of real-time PCR methods for detection of *Salmonella enterica* and *Escherichia coli* 0157:H7, and introduction of a general internal amplification control. *Journal of Microbiological Methods*. **59**, 337 – 349.
- Knight, S. M. 2002. Managing early-drilled second wheats to minimise the impact of take-all. *Home-Grown Cereals Authority Project. Report No. 255*.
- Kollmorgen, J. F., Griffiths, J. B. and Walsgott, D. N. 1983. The effects of various crops on the survival and carry-over of the wheat take-all fungus *Gaeumannomyces graminis* var. *tritici*. *Plant Pathology*. **32**, 73-77.
- Kuske, C. R., Banton, K. L., Adorada, D. L., Stark, P. C., Hill, K. K. and Jackson, P. J. 1998. Small-scale sample preparation method for field PCR detection of microbial cells and spores in soil. *Applied and Environmental Microbiology*. **64**, 2463 – 2472.

- Landschoot, P. J. and Jackson, N. 1989. *Gaeumannomyces incrustans* sp. nov., a root-infecting hyphopodiate fungus from grass roots in the United States. *Mycological Research*. **93**, 55 – 58.
- Lees, A. K., Cullen, D. W., Sullivan, L. and Nicolson, M. J. 2002. Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. *Plant Pathology*. **51**, 293 – 302.
- Litvintseva, A. P. and Henson, J. M. 2002a. Cloning, characterization, and transcription of three laccase genes from *Gaeumannomyces graminis* var. *tritici*, the take-all fungus. *Applied and Environmental Microbiology*. **68**, 1305 – 1311.
- Litvintseva, A. P. and Henson, J. M. 2002b. Characterization of two laccase genes of *Gaeumannomyces graminis* var. *graminis* and their differential transcription in melanin mutants and wild type. *Mycological Research*. **106**, 808 – 814.
- Liu, C. H., Shang, H. S. and Tan, R. X. 2000. Infection process of take-all causing fungi (*Gaeumannomyces graminis* var. *tritici*) on wheat (*Triticum aestivum*) and oat (*Avena sativa*) roots. *Indian Journal of Agricultural Sciences*. **70**, 23 – 27.
- Lochel, A. M., Wenz, M., Russell, P. E. Buschhaus, E., Evans, P. H., Cross, S., Puhl, T. and Bardsley, E. 1998. Root protection using fluquinconazole: a new approach to controlling cereal take-all. **In: The 1998 Brighton Conference – Pests and Diseases**. **1**, 89 – 96. Farnham, Surrey: British Crop Protection Council.
- Martyniuk, S. 1998. Pectolytic enzymes activity and pathogenicity of *Gaeumannomyces graminis* var. *tritici* and related Phialophora-like fungi. *Plant and Soil*. **107**, 19 – 23.

McCarthy, L. B. and Lucas, L. T. 1989. *Gaeumannomyces graminis* associated with Spring Dead Spot of Bermudagrass in the Southeastern United States. *Plant Disease*. **73**, 659 – 661.

McSpadden Gardener, B. B. and Weller, D. M. 2001. Changes in population of rhizosphere bacteria associated with take-all disease of wheat. *Applied and Environmental Microbiology*. **67**, 4414-4425.

Miller, D. N. 2001. Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soil and sediments. *Journal of Microbiological Methods*. **44**, 49 – 58.

Miller, D. N., Bryant, J. E., Madsen, E. L. and Ghiorse, W.C. 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Applied and Environmental Microbiology*. **65**, 4715 – 4724.

Mullis, K. B. and Faloona, F. A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Method in Enzymology*. **155**, 335-350.

Neate, S. M. 1987. Plant debris in soil as a source of inoculum of *Rhizoctonia* in wheat. *Transactions of the British Mycological Society*. **88**, 157-162.

Nicholson, P. and Parry, D. W. 1996. Development and use of a PCR assay to detect *Rhizoctonia cerealis*, the cause of sharp eyespot in wheat. *Plant Pathology*. **45**, 872 – 883.

Nicholson, P., Lees, A. K., Maurin, N., Parry, D. W. and Rezanoor, H. N. 1996. Development of a PCR assay to identify and quantify *Microdochium nivale* var. *nivale* and

*Microdochium nivale* var. *majus* in wheat. *Physiological and Molecular Plant Pathology*.

**48**, 257 – 271.

Nicholson, P., Rezanoor, H. N., Simpson, D. R. and Joyce D. 1997. Differentiation and quantification of the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis* using a PCR assay. *Plant Pathology*. **46**, 842 – 856.

O'Donnell, K., Kistler, H. C., Tacke, B. K. and Casper, H. H. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proceedings of the National Academy of Sciences of the United States of America*. **97**, 7905-7910.

Ogram, A., Sayler, G. S. and Barkay, T. 1987. The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods*. **7**, 57-66.

Ophel-Keller, K., Engel, B. and Heinrich, K. 1995. Specific detection of *Gaeumannomyces graminis* in soil using polymerase chain reaction. *Mycological Research*. **99**, 1385-1390.

Osbourn, A., Clarke, B. R., Dow, J. M. and Daniels, M. J. 1991. Partial characterization of avenacinase from *Gaeumannomyces graminis* var. *avenae*. *Physiological and Molecular Plant Pathology*. **38**, 301 – 312.

Osbourn, A., Clarke, B. R., Lunness, P., Scott, P. R. and Daniels, M. J. 1994. An oat species lacking avenacin is susceptible to infection by *Gaeumannomyces graminis* var. *tritici*. *Physiological and Molecular Plant Pathology*. **45**, 457 – 467.

- Parry, D. W. and Nicholson, P. 1996. Development of a PCR assay to detect *Fusarium poae* in wheat. *Plant Pathology*. **45**, 383 – 391.
- Penrose, L. 1985. Evidence for resistance in wheat cultivars grown in sand culture to the take-all pathogen, *Gaeumannomyces graminis* var. *tritici*. *Annals of Applied Biology*. **107**, 105 – 108.
- Penrose, L. D. J. and Neate, S. M. 1994. Resistance to *Gaeumannomyces graminis* in wheat genotypes grown in field environments and sand culture. *Soil Biology and Biochemistry*. **26**, 719 – 726.
- Polley, R. W. and Clarkson, J. D. S. 1980. Take-all severity and yield in winter wheat: relationship established using a single plant assessment. *Plant Pathology*. **29**, 110-116.
- Polley, R. W. and Thomas, M. R. 1991. Surveys of diseases of winter wheat in England and Wales, 1976-1988. *Annals of Applied Biology*. **119**, 1 – 20.
- Rachdawong, S., Cramer, C. L., Grabau, E. A., Stomberg, V. K., Lacy, G. H. and Stromberg, E. L. 2002. *Gaeumannomyces graminis* vars. *avenae*, *graminis* and *tritici* identified using PCR amplification of avenacinase-like genes. *Plant Disease*. **86**, 652-660.
- Raeder, U. and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology*. **1**, 17-20.
- Reis, E. M., Cook, R. J. and McNeal, B. L. 1982. Effect of mineral nutrition on take-all of wheat. *Phytopathology*. **72**, 224 – 229.

Reis, E. M., Cook, R. J. and McNeal, B. L. 1983. Elevated pH and associated reduced trace-nutrient availability as factors contributing to take-all of wheat upon soil liming. *Phytopathology*. **73**, 411 – 413.

Roget, D. K. and Rovira, A. D. 1991. The relationship between incidence of infection by the take-all fungus (*Gaeumannomyces graminis* var. *tritici*), rainfall and yield of wheat in South Australia. *Australian Journal of Experimental Agriculture*. **31**, 509 – 513.

Rosser, W. R. and Chambers, B.L. 1968. Cereal diseases and their effects on intensive wheat cropping in the East Midland region, 1963-65. *Plant Pathology*. **61**, 51-60.

Russell, P. E., Percival, A., Coltman, P. M. and Green, D. E. 1992. Fluquinconazole, a novel broadspectrum fungicide for foliar application. **In:** *Brighton Crop Protection Conference - Pests and Diseases - 1992*. **1**, 411 – 418. Farnham, Surrey: British Crop Protection Council.

Saiki, R. K. 1992. The design and optimisation of the PCR. **In:** (H. A. Erlich. *ed.*) *PCR Technology. Principles and applications for DNA amplification*. New York, USA, W. H. Freeman and Company. pp 7-16.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. 1985. Enzymatic amplification of  $\beta$ -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science*. **230**, 1350-1354.

Biology Workbench. San Diego Supercomputer Centre. <http://workbench.sdsc.edu/>  
[Accessed 12/10/01]



- Schesser, K., Luder, A. and Henson, J. M. 1991. Use of polymerase chain reaction to detect the take-all fungus, *Gaeumannomyces graminis*, in infected wheat plants. *Applied and Environmental Microbiology*. **57**, 553-556.
- Schoeny, A. and Lucas, P. 1999. Modelling of take-all epidemics to evaluate the efficacy of a new seed-treatment fungicide on wheat. *Phytopathology*. **89**, 954 – 961.
- Schoeny, A., Lucas, P and Jeuffroy, M-H. 1998. Influence of the incidence and severity of take-all of winter wheat on yield losses and responses to different nitrogen fertilisations. **In: The 1998 Brighton Conference – Pests and Diseases**. **1**, 83 – 88. Farnham, Surrey: British Crop Protection Council.
- Schoeny, A., Jeuffroy, M-H. and Lucas, P. 2001. Influence of take-all epidemics on winter wheat yield formation and yield loss. *Phytopathology*. **91**, 694 – 701.
- Schoeny, A., Devienne-Baret, F., Jeuffroy, M-H. and Lucas, P. 2003. Effect of take-all root infections on nitrate uptake in winter wheat. *Plant Pathology*. **52**, 52 – 59.
- Scott, P. R. 1981. Variation in host susceptibility. **In: M. J. C. Asher and P. J. Shipton. eds. *Biology and Control of Take-all***. London. Academic Press. pp. 175-198.
- Shipton, P. J. 1972. Take-all in spring-sown cereals under continuous cultivation: disease progress and decline in relation to crop succession and nitrogen. *Annals of Applied Biology*. **71**, 33 – 46.
- Shou, J. P. 1981. Morphology and cytology of the infection process. **In: M. J. C. Asher and P. J. Shipton. eds. *Biology and Control of Take-all***. London. Academic Press. pp. 175-198.

Smalla, K., Cresswell, N., Mendonca-Hagler, L. C., Wolters, A. and van Elsas, J. D. 1993. Rapid extraction protocol from soil for polymerase chain reaction-mediated amplification. *Journal of Applied Bacteriology*. **74**, 78 – 85.

Smiley, R. W. and Cook, R. J. 1973. Relationship between take-all of wheat and rhizosphere pH in soils fertilized with ammonium vs. nitrate-nitrogen. *Phytopathology*. **63**, 882 – 890.

Spink, J. H., Blake, J. J., Foulkes, J., Pillinger, C. and Paveley, N. 2002. Take-all in winter wheat. Effects of silthiofam (Latitude) and other management factors. *Home-Grown Cereals Authority Project. Report No. 268*.

Stach, J. E. M., Bathe, S., Clapp, J. P. and Burns, R. G. 2001. PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. *FEMS Microbiology Ecology*. **36**, 139 – 151.

Steffan, R. J. and Atlas, R. M. 1991. Polymerase chain reaction: Applications in environmental microbiology. *Annual Review of Microbiology*. **45**, 137-161.

Steffan, R. J., Goksoyr, J., Bej, A. K. and Atlas, R. M. 1988. Recovery of DNA from soil and sediments. *Applied and Environmental Microbiology*. **54**, 2908 – 2915.

Stevenson, F. J. 1994. Organic matter reactions involving metal ions in soil. **In:** F. J. Stevenson eds. *Humus Chemistry. Genesis, Composition, Reaction*. New York, USA. John Wiley and Sons, Inc. pp 394 – 401.

- Tan, M. K. and Wong, P. T. W. 1996. Group I introns in 26S rRNA genes of *Gaeumannomyces graminis* as possible indicators of host specificity of *G. graminis* varieties. *Mycological Research*. **100**, 337 – 342.
- Tan, M. K., Wong, P. T. W. and Holley, M. P. 1994. Characterization of nuclear ribosomal DNA (rDNA) in *Gaeumannomyces graminis* and correlation of rDNA variation with *G. graminis* varieties. *Mycological Research*. **98**, 553 – 561.
- Tebbe, C. C. and Vahjen, W. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and yeast. *Applied and Environmental Microbiology*. **59**, 2657 – 2665.
- Thornton, R. T., Dewey, F. M. and Gilligan, C. A. 1997. Production and characterization of a monoclonal antibody raised against surface antigens of *Gaeumannomyces graminis* var. *tritici*: evidence for an extracellular polyphenol oxidase. *Phytopathology*. **87**, 123 – 131.
- Towner, P. 2000. Purification of DNA. **In:** T. A. Brown ed. *Essential Molecular Biology*. Oxford University Press. pp 55-67.
- Tsai, Y. L. and Olson, B. H. 1992a. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Applied and Environmental Microbiology*. **58**, 754 – 757.
- Tsai, Y. L. and Olson, B. H. 1992b. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Applied and Environmental Microbiology*. **58**, 2292 – 2295.

Turner, E. M. 1940. *Ophiobolus graminis* Sacc. var. *avenae* var. nov., as the cause of take-all or whiteheads of oats in Wales. *Transactions of the British Mycological Society*. **24**, 269-281.

Ulrich, K., Augustin, C. and Werner, A. 2000. Identification and characterization of a new group of root-colonizing fungi within the *Gaeumannomyces-Phialophora* complex. *New Phytologist*. **145**, 127 – 135.

Volossiuk, T., Robb, E. J. and Nazar, R. N. 1995. Direct DNA extraction for PCR-mediated assays of soil organisms. *Applied and Environmental Microbiology*. **61**, 3972 - 3976

von Arx, J. A. and Oliver, D. L. 1952. The taxonomy of *Ophiobolus graminis* Sacc. *Transactions of the British Mycological Society*. **35**, 29-33.

Walker, J. 1972. Type studies on *Gaeumannomyces graminis* and related fungi *Transactions of the British Mycological Society*. **58**, 427-457.

Walker, J. 1980. *Gaeumannomyces*, *Linocarpon*, *Ophiobolus* and several other genera of scolecospored ascomycetes and *Phialophora* conidial states, with a note on hyphopodia. *Mycotaxon*. **11**, 1 – 129.

Ward, E. 1995. Improved polymerase chain reaction (PCR) detection of *Gaeumannomyces graminis* including a safeguard against false negatives. *European Journal of Plant Pathology*. **101**, 561-566.

- Ward, E. and Bateman, G. L. 1999. Comparison of *Gaeumannomyces* - and *Phialophora*-like fungal pathogens from maize and other plants using DNA methods. *New Phytologist*. **141**, 323-331.
- Ward, E. and Gray, R. M. 1992. Generation of a ribosomal DNA probe by PCR and its use in identification of fungi within the *Gaeumannomyces* - *Phialophora* complex. *Plant Pathology*. **41**, 730 – 736.
- Wenz, M., Russell, P. E., Lochel, A. M., Buschhaus, H., Evans, P. H., Bardsley, E., Petit, F. and Puhl, T. 1998. Seed treatment with fluquinconazole for control of cereal take-all, foliar and seed-borne diseases. **In: The 1998 Brighton Conference – Pests and Diseases**. **3**, 907 – 912. Farnham, Surrey: British Crop Protection Council.
- Werker, A. R. and Gilligan, C. A. 1990. Analysis of the effects of selected agronomic factors on the dynamics of the take-all disease of wheat in field plots. *Plant Pathology*. **39**, 161 – 177.
- Weste, G. 1972. The process of root infection by *Ophiobolus graminis*. *Transactions of the British Mycological Society*. **59**, 133 – 147.
- Weste, G. and Thrower, L. B. 1963. Production of perithecia and microconidia in culture by *Ophiobolus graminis*. *Phytopathology*. **53**, 354.
- Wetzel III, H. C., Dernoeden, P. H. and Millner, P. D. 1996. Identification of darkly pigmented fungi associated with turfgrass roots by mycelial characteristics and RAPD-PCR. *Plant Disease*. **80**, 359 – 364.

- Whisson, D. L., Herdina and Francis, L. 1995. Detection of *Rhizoctonia solani* AG-8 in soil using a specific DNA probe. *Mycological Research*. **99**, 1299-1302.
- White, T. J., Burns, S., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. **In:** Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. eds. *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, CA, USA. Pp 315-322.
- Wilkinson, H. T., Cook, R. J. and Alldredge, J. R. 1985. Relationship of inoculum size and concentration to infection of wheat roots by *Gaeumannomyces graminis* var. *tritici*. *Phytopathology*. **75**, 98 – 103.
- Wong, P. T. W. 2002. *Gaeumannomyces wongoonoo* sp. nov., the cause of a patch disease of buffalo grass (St Augustine grass). *Mycological Research*. **106**, 857-862.
- Yao, J. M. 1992. A new variety of the pathogen of maize take-all. *Acta Mycologica Sinica*. **11**, 99 – 104.
- Yeates, J. S. 1986. Ascospore length of Australian isolates of *Gaeumannomyces graminis*. *Transactions of the British Mycological Society*. **86**, 131 – 136.
- Zadoks, J. C., Chang, T. T. and Kondak, C. F. 1974. A decimal code for the growth stages of cereals. *Weed Research*. **14**, 412-421.
- Zhou, J., Bruns, M. A. and Tiedje, J. M. 1996. DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology*. **62**, 316 – 322.

Zimmermann, K. and Mannhalter, J. W. 1996. Technical aspects of quantitative competitive PCR. *BioTechniques*. **21**, 268-278.

# **Appendices**



# Appendix 1: List of isolates used to test HAGG primers in Chapter 4. All

isolates were isolated during this project unless otherwise stated.

Name	Host	Location	Date	Identified as <i>G.</i> <i>graminis</i> by morphology (Y/N)	Reaction with HAGG primers (+/-)
NOR02/03	wheat	Norfolk	2002	Y	+
NOR02/04	wheat	Norfolk	2002	Y	+
NOR02/05	wheat	Norfolk	2002	Y	+
NOR02/06	wheat	Norfolk	2002	Y	+
NOR02/09	wheat	Norfolk	2002	Y	+
NOR02/11	wheat	Norfolk	2002	Y	+
NOR02/13	wheat	Norfolk	2002	Y	+
NOR02/15	wheat	Norfolk	2002	Y	+
NOR02/16	wheat	Norfolk	2002	Y	+
NOR02/19	wheat	Norfolk	2002	Y	+
NOR02/20	wheat	Norfolk	2002	Y	+
NOR02/22	wheat	Norfolk	2002	Y	+
NOR02/26	wheat	Norfolk	2002	Y	+
NOR02/27	wheat	Norfolk	2002	Y	+
NOR02/29	wheat	Norfolk	2002	Y	+
NOR02/30	wheat	Norfolk	2002	Y	+
OXF03/01	wheat	Oxfordshire	2003	Y	+
OXF03/02	wheat	Oxfordshire	2003	Y	+
OXF03/03	wheat	Oxfordshire	2003	Y	+
OXF03/04	wheat	Oxfordshire	2003	Y	+
OXF03/05	wheat	Oxfordshire	2003	Y	+
OXF03/06	wheat	Oxfordshire	2003	Y	+
OXF03/07	wheat	Oxfordshire	2003	Y	+
OXF03/08	wheat	Oxfordshire	2003	Y	+
OXF03/09	wheat	Oxfordshire	2003	Y	+
OXF03/10	wheat	Oxfordshire	2003	Y	+
OXF03/11	wheat	Oxfordshire	2003	Y	+
OXF03/12	wheat	Oxfordshire	2003	Y	+
OXFN03/01	wheat	Oxfordshire	2003	N	-
OXFN03/02	wheat	Oxfordshire	2003	N	-
OXFN03/03	wheat	Oxfordshire	2003	N	-
HERT03/01	wheat	Hertfordshire	2003	N	-
HERT03/02	wheat	Hertfordshire	2003	Y	+
NYOR03/01	wheat	N. Yorkshire	2003	Y	+
NYOR03/02	wheat	N. Yorkshire	2003	Y	+
NYOR03/03	wheat	N. Yorkshire	2003	Y	+
ESSE03/01	wheat	Essex	2003	Y	+
ESSE03/02	wheat	Essex	2003	Y	+
WORC03/01	wheat	Worcestershire	2003	N	-
WORC03/02	wheat	Worcestershire	2003	N	-
EVE03/01	wheat	Evesham	2003	Y	+
DUR03/01	wheat	Durham	2003	Y	+

COD04/01	wheat	Codeby	2004	Y	+
COD04/02	wheat	Codeby	2004	Y	+
WSS04/01	wheat	W. Sussex	2004	Y	+
WSS04/02	wheat	W. Sussex	2004	Y	+
NEO04/01	wheat	St. Neots	2004	N	-
BAG04/01	wheat	Bagworth	2004	N	-
BAG04/01	wheat	Bagworth	2004	N	-
ELY04/01	wheat	Cambridgeshire	2004	N	-
88/10-1 <sup>a</sup>	wheat	Bedfordshire	1988	Y	+
90/2-4 <sup>a</sup>	wheat	Bedfordshire	1990	Y	+
92/3-2 <sup>a</sup>	triticale	Hertfordshire	1992	Y	+
92/15-4A <sup>a</sup>	wheat	Hertfordshire	1992	Y	+
92/24-2 <sup>a</sup>	Barley	Hertfordshire	1992	Y	+
92/49-2A <sup>a</sup>	wheat	Cambridgeshire	1992	Y	+
93/17-2 <sup>a</sup>	barley	Cumbria	Not known	Y	+
93/18-1 <sup>a</sup>	barley	Cumbria	Not known	Y	+
ABL2 <sup>a</sup>	turf	Yorkshire	1990	Y	+
Lawn <sup>a</sup>	Not known	Not known	Not known	Y	+
YZ2 <sup>a</sup>	Not known	Not known	Not known	Y	+
<i>Fusarium graminearum</i> <sup>b</sup>	Wheat	Not known	Not known	N	-
<i>F. poae</i> <sup>b</sup>	Wheat	Not known	Not known	N	-
<i>F. avenaceum</i> <sup>b</sup>	Wheat	Not known	Not known	N	-
<i>F. culmorum</i> <sup>b</sup>	Wheat	Not known	Not known	N	-
<i>F. langsethiae</i> <sup>b</sup>	Oats	Not known	Not known	N	-
<i>Oailimacula yallundae</i> <sup>c</sup>	Wheat	Not known	Not known	N	-
<i>O. tapesia</i> <sup>c</sup>	Wheat	Not known	Not known	N	-

<sup>a</sup> Isolates supplied by G. Bateman from Rothamsted

<sup>b</sup> Isolates from the Harper Adams University College culture collection

<sup>c</sup> Isolates supplied by R. Ray, HAUC.

Appendix 2: List of primers, and their sequences. Underlined is the part of the linker primer which is the same as the HAGG primer.

Primer	Sequence	Chapter
ITS 4	ttc ttc gct tat tga tat gc	2
ITS 5	gga agt aaa agt cgt aac aag g	2
ONI670/F	gtt gct cat gcc cct tat ta	3
ONI670/R	tga ggt cgc cga tgg tgc	3
SP6+3	tta ggt gac act ata gaa tag tc	3
KS1/F	tac ggc tgt acc gca tga tga tct act a	4
KS2/R	atg agc cca gag gtc ccg tca tca aaa	4
pGt1	ttg ctt cgg cgg acg atg gc	4
pGt2	gtt act gcg ttc agg gtc ctg	4
NS5	aac tta aag gaa ttg acg gaa	4
GGA-RP	ttt gtg tgt gac cat ac	4
GGT-RP	tgc aat ggc ttc gtg aa	4
Gga	acg gcg gtg gat ggc aag ac	4
Ggg	cac ccc cgg tcc ctg cgt a	4
Ggt	tcc tcg gcc ccg taa ttg gc	4
AVE/R	tgc tca tgg tgg ttc ctg cg	4
HAGG/F	aac tcc aac ccc tgt gaa ca	4
HAGG/R1	cga gac cgc cga tgt tc	4
HAGG/R2	tac tgc gtt cag ggt cct g	4
HAGG/R3	ttt acc gcg agt tac tgc gt	4
526F	tgg cag cag cac aa	4
526R	tgg cga agc aga cc	4
HAGG/FL	<u>cct gtg aac</u> atg gca gca gca caa	4
HAGG/R3L	<u>gtt act gcg ttg</u> gcg aag cag acc	4

### Appendix 3: Details of fields sampled in Chapter Four.

Field	Soil Type	Seed treatment	Cultivar	Date planted
DIT	Clay loam	Jockey	Not known	3/10/03
PAV	Loamy clay	Latitude	Enstein	13/10/03
PAVE	Loamy clay	Latitude	Enstein	13/10/03
PRE	Loam	SPD	Napier	Late October
WAL	Clay loam	SPD	Consort	Early October
WIN	Loam	SPD	Tanker	Early October
BED	Clay	SPD	Consort	15/10/03
HALL	Sandy loam	Latitude	Napier	Early October
HALN	Sandy loam	SPD	Napier	Early October
HUNT1	Sandy clay	Beret Gold	Napier	20/09/03
HUNT2	Sandy clay	Beret Gold	Tanker	21/09/03
RUT1	Silty loam	SPD	Gladiator	15/10/03
RUT2	Silty loam	SPD	Gladiator	15/10/03

SPD – Single purpose dressing (not take-all active)

Appendix 4: Agronomy of the field experiment performed at Much Wenlock, Shropshire in 2003/2004.

Date	Chemical	Active ingredient	Concentration	Rate	Manufacturer
17/11/03	Cyperkill 25 Panther	cypermethrin	250 g l <sup>-1</sup>	0.025 l/ha	Chimac-
		diflufenican	50 g l <sup>-1</sup>	0.5 l/ha	Agriphar SA
	IPU minrinse	Isoproturon	500 g l <sup>-1</sup>		Bayer
		isoproturon	80% w/w	2 l/ha	CropScience Ltd
					Makhteshim-Agan (UK) Ltd
18/03/04	Extran	ammonium nitrate	33.5% w/w	125 kg/ha	Yara UK Ltd
9/04/04	Cheetah Super	fenoxaprop-P-ethyl	55 g l <sup>-1</sup>	0.75 l/ha	Bayer
		chloromequat	730 g l <sup>-1</sup>	2.3 l/ha	CropScience Ltd
	Hive Starane	fluroxypyr	288 g l <sup>-1</sup>	1 l/ha	Nufarm UK Ltd
					Dow
					AgroSciences
10/04/04	Extran	ammonium nitrate	33.5% w/w	250 kg/ha	Yara UK Ltd
1/05/04	Extran	ammonium nitrate	33.5% w/w	250 kg/ha	Yara UK Ltd
10/05/04	Joules Landmark	chlorothalonil	500 g l <sup>-1</sup>	1 l/ha	Nufarm UK
		epoxiconazole	125 g l <sup>-1</sup>	0.5 l/ha	Ltd
		kresoxim-methyl	125 g l <sup>-1</sup>		BASF plc
26/05/04	Opus Twist	epoxiconazole	125 g l <sup>-1</sup>	0.5 l/ha	BASF plc
		trifloxystrobulin	50% w/w	0.8 l/ha	Bayer
					CropScience Ltd
5/08/04	Roundup	glyphosphate	360 g l <sup>-1</sup>	3 l/ha	Monsanto plc